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A FEMINIZING ADRENAL TUMOR CAUSING GYNECOMASTIA IN A BOY OF FIVE YEARS CONTRASTED WITH A VIRILIZING TUMOR IN A FIVE-YEAR-OLD GIRL 111
Lawson Wilkins

THE EFFECT OF STARVATION ON URINARY 17-KETOSTEROID EXCRETION 133
Richard L. Landau, Kathryn Knowlton, Dolores Anderson, Minnie B. Brandt and Allan T. Kenyon

A COMPARISON OF ORAL AND VAGINAL EPITHELIAL SMEARS . . . 146
Daniel E. Ziskin and Ruth Moulton

CHEMICAL ASSAY FOR "CORTIN" 166
William H. Daughaday, Herbert Jaffe and Robert H. Williams

HORMONE SECRETION BY HUMAN PLACENTA GROWN IN TISSUE CULTURE 175
H. L. Stewart, Jr., Machteld E. Sano and Thaddeus L. Montgomery

ENDOCRINE REVIEWS 189
Willard O. Thompson

STEROID NOMENCLATURE 190
Harold L. Mason

OBITUARY: Fred Conrad Koch (1876-1948) 207

ANNOUNCEMENT OF THE 1948 MEETING OF THE ASSOCIATION 208

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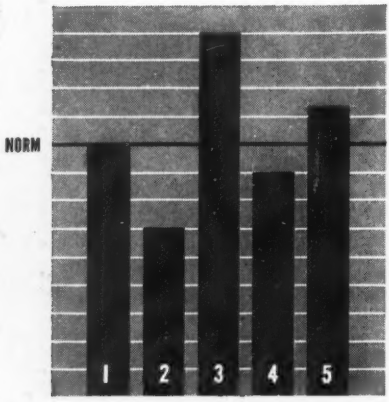

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A FEMINIZING ADRENAL TUMOR CAUSING GYNECOMASTIA IN A BOY OF FIVE YEARS CONTRASTED WITH A VIRILIZING TUMOR IN A FIVE-YEAR-OLD GIRL

CLASSIFICATION OF SEVENTY CASES OF ADRENAL TUMOR IN CHILDREN ACCORDING TO THEIR HORMONAL MANIFESTATIONS AND A REVIEW OF ELEVEN CASES OF FEMINIZING ADRENAL TUMOR IN ADULTS*

LAWSON WILKINS, M.D.

From the Department of Pediatrics, Johns Hopkins University School of Medicine, and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland.

TWO children, a boy and a girl, each 5 years old, with marked changes toward the adult characteristics of the opposite sex, were studied at about the same time in the Endocrine Clinic of the Harriet Lane Home. In each case an encapsulated adenoma of the right adrenal was successfully removed. The cases are of interest as examples of the difference in the hormonal effects of adrenal tumors and because hitherto no instance of gynecomastia due to adrenal tumor has been reported in a boy before puberty.

CASE REPORTS

Case 1. Donald G. (H.L.H. A 31153) came to our attention July, 1943, at the age of 4 years and 8 months because of large, well developed breasts. During the neonatal period hyperplasia of the breasts so common among newborns was not noted, but at the

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age of 6 months the breasts became enlarged and attained their present state of development by the age of 1 or 2 years. There had been no tenderness and no secretion. Except for the breasts, the boy's growth, development and general health had been normal in every way. He behaved like a normal boy and showed no feminine traits of personality.

At the time he was examined, he was only 1 inch taller than the average and his skeletal proportions were entirely normal. However, his epiphysial development was that of a 10-year old boy, and his dental development was slightly advanced (Fig. 1).

FIG. 1a. *Case 1:* D.G. age 5 years. Before operation. Note the well developed breasts and nipples and normal male genitalia.
FIG. 1b. *Case 1:* D.G. age 8¼ years. Three years after operation. Breasts much smaller. No signs of puberty.

The breasts were conical and measured 9 cm. in diameter at the base. The central area of 5 cm. diameter felt like normal glandular structure. The areolae were well developed but did not show much pigment. The nipples were small. There were well marked pectoral and circumareolar veins. No secretion could be expressed. The penis was normal for the patient's age and the testes were of normal size and consistency, measuring 2×1.2 cm. The prostate was definitely enlarged for his age, about one-third the adult size and was of unusually firm fibrous consistency. There was no pubic hair. The skin was normal and showed no unusual pigmentation or seborrhea.

The general examination was essentially negative. Palpation of the abdomen revealed no masses. X-rays of the bones, skull and lungs showed no tumor metastases. Intravenous pyelograms showed a double renal pelvis and a bifid ureter on the right side.

Above the right kidney and apparently not connected with it, there was a calcified area 1 cm. in diameter visible on both the flat X-ray plate and the intravenous pyelogram. Urine examination and P.S.P. showed no abnormality. The intradermal tuberculin test was negative. Blood counts were normal. Chemical examination of the serum showed the concentration of N.P.N. to be 19 mg. per cent, calcium 11.9 mg. per cent, phospho-

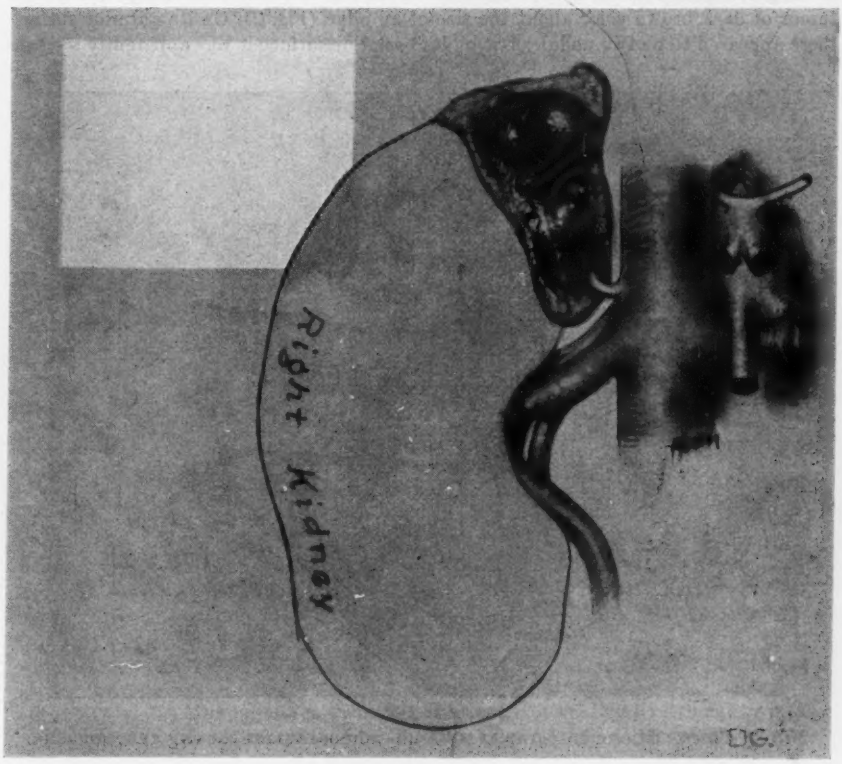

FIG. 2. *Case 1:* Drawing of adrenal tumor causing gynecomastia.

rus 5.3 mg. per cent, phosphatase 8.7 Bodansky units, chloride 101 millequivalents, and cholesterol 132 mg. per cent. Glucose tolerance and insulin sensitivity tests were negative. The Kepler-Powers diuresis test for adrenal function revealed no abnormality. The 17-ketosteroid excretion was 4.1 mg. per 24 hours. Assay of urinary estrogen (Dr. Delfs) showed an excretion of 5 rat units per 24 hours which is only slightly greater than normal for even an adult male. Prégnandiol was not detected in the urine. An assay of F.S.H. was negative in a titer of 6.5 mouse units per 24 hours.

Because of the occurrence of gynecomastia at this early period of life, and because of the excretion of estrin in the urine, it was believed the boy probably had a tumor producing an estrogenic substance. The possibility of a testicular tumor, an embryonic tumor somewhere in the abdominal cavity or a tumor of the adrenal was considered.

The only suggestion as to its location was the calcified shadow in the right suprarenal region.

It was felt that a transperitoneal incision which would permit exploration of the abdomen as well as approach to the adrenal was indicated. This was performed by Dr. Alfred Blalock and Dr. Hugh Jewett November 16, 1943. No abnormality was found in the pelvis or abdominal cavity. Exposure of the right adrenal immediately revealed a tumor of dark brown color about the size of an olive (Fig. 2). On its anterior surface there appeared to be two nodules but on its posterior surface it was apparently a single

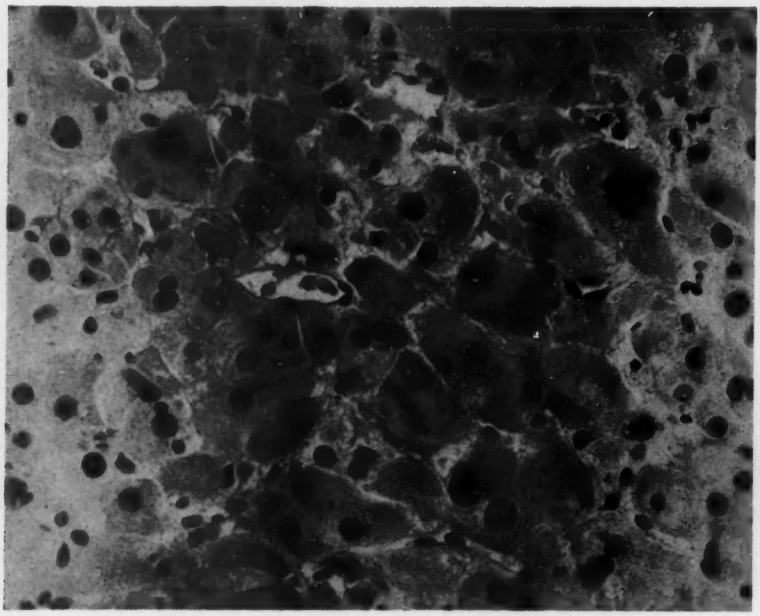

FIG. 3. *Case 1:* Microphotograph (×400) of adrenal tumor causing gynecomastia.

mass. The tumor was removed together with some adrenal tissue, leaving only a small portion of the upper pole of the adrenal. The left adrenal was carefully examined and appeared normal.

Pathological Examination by Dr. Sam Blackman: "The specimen removed consisted of adrenal gland and tumor measuring 3×2 cm. Occupying a large proportion of this and arising slightly closer to one end than the other, there is a trilobular firm tumor which on one side reveals two nodules projecting from the surface of the adrenal and on the other side, a single larger lobule. One lobule is calcified and is cut with difficulty. The rest of the tumor is soft and brown in color.

Microscopic Examination: (Fig. 3). The tumor is separated from the normal adrenal by a fibrous capsule. There are calcified areas in the capsule and scattered calcified areas in the tumor itself. The section fixed in Vandegrift's solution shows very large cells although some are only moderately large. The cells vary in shape a good deal. They have

deeply eosinophilic coarse granular cytoplasm. Some of the cytoplasm has dropped out. The outline of every cell is sharp, so that it appears almost as if there were a distinct membrane around each cell. The nuclei are mostly round and vary greatly in size. Some are huge, hyperchromatic nuclei; many contain nucleoli. Tissue fixed in Zenker's solution shows the same large cells of irregular shape and the same variable nuclei, many of which are enormous and extremely hyperchromatic. There are no mitotic figures. In

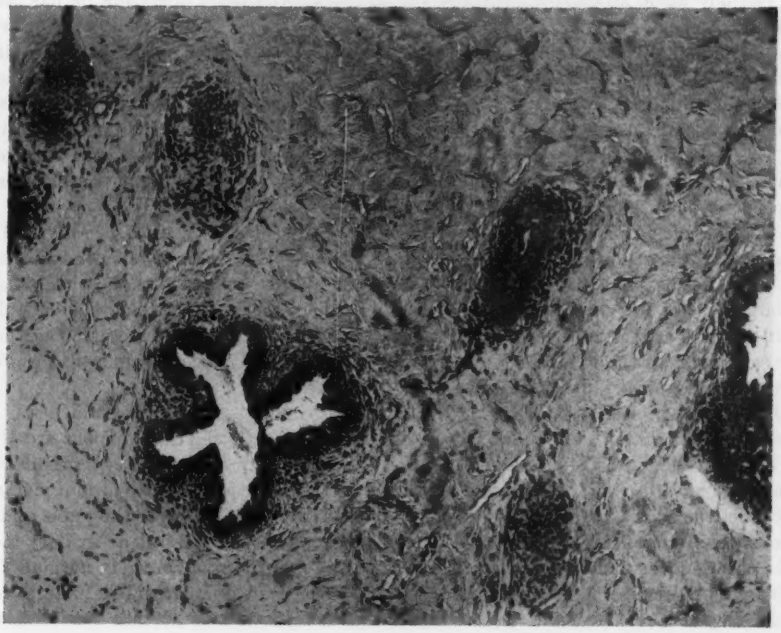

FIG. 4. *Case 1:* Microphotograph (×90) of breast biopsy. Relatively slight hyperplasia of ducts but marked proliferation of periductal fibrous stroma.

the Zenker-fixed tissue, however, the outlines of the cells are less distinct, and the cytoplasm has not dropped out. Moreover, the appearance of a cell membrane is not present in this tissue at all. Many of the cells in the Zenker-fixed tissue contain relatively large droplets of yellowish brown material which is highly refractive and accounts for the brownish color of the gross specimen. These pigmented drops were evidently dissolved out in the Vandegrift's fixed tissue, probably by the acetone. They are entirely different from the minute, more brown granules of pigment that are found in tumors and hyperplasias of the adrenogenic zone of the adrenal cortex (1). Sections fixed in Zenker's fluid and stained with iron-hematoxylin show hematoxylin-stained granules and droplets in many of the cells, such as are found in the prenatal zone of the adrenal cortex. These did not form complete circles in the cells like the pigment granules and their preceding mitochondria which occur in the reticular zone cells of the adrenal cortex. With ponceau-fuchsin stain the protoplasm of the cells appears granular and stains deeply red. The cells

of this tumor are identical with microscopical cell masses which we have found in the prenatal zone of the adrenal cortex of infants at autopsy.

Biopsy of breast (Fig. 4) shows some hyperplasia of the duct epithelium and marked periductal hyperplasia of the connective tissue. The connective tissue appears old and hyaline and contains few nuclei. It is so widespread that it no longer shows any particular periductal arrangement, as is seen in early cases of gynecomastia. It is evident that the main mass of the enlarged breast consists of this hyaline connective tissue."

The boy made an uneventful recovery. In the 4 years since operation he has been examined repeatedly in the Endocrine Clinic. His health has been good and his growth and development normal. The breasts have decreased slowly but progressively in size. They are still quite noticeable but measure only 3 to 4 cm. in diameter and are more soft and flabby. In June, 1947, at the age of 8 years and 9 months, he was 50.3 inches tall, which is within average range for this age. The bone age was about 9½ years. According to Dr. Jewett, the prostate had decreased somewhat in size but was still enlarged. There was almost a stony induration of the left lobe, whereas the right was of normal consistency. No calcification was demonstrated by X-ray.

Case 2. Jane C. (H.L.H. A 33040) was 5½ years old when she was first seen by us. The mother and the attending physician were certain that her genitalia were entirely normal at birth. At the age of 5 years, in May, 1943, the mother noticed that there was slight enlargement of the clitoris which grew rapidly from then on. About September, pubic hair began to appear and grew rapidly thereafter. Her voice became deeper, her features coarser, and her skin became oily and showed a little acne. Within a few months she grew 3 inches and gained 10 lbs., becoming more muscular.

In November, 1943, at the age of 5½ years her height was 44.6 inches which was 1½ inches taller than the average for her age (Fig. 5). The epiphysial development was that of 7 years. The musculature was well developed. Her features were a little more mature than normal for her age. There were seborrhea and slight acne but no abnormal pigmentation of the skin. There was a pronounced growth of coarse hair over the pubis and in the perianal region. There was some dark down on the upper lip but no axillary hair. The clitoris measured 3 cm. × 1 cm. The vulval configuration was entirely normal. The labia majora were somewhat hypertrophied and pigmented, while the labia minora were undeveloped and of preadolescent type. The meatus of the urethra was situated in the usual position near the base of the clitoris, and the vaginal orifice admitted a finger. In this respect she differed from female pseudohermaphrodites in whom the occurrence of androgenic hyperplasia of the adrenals in early embryonic life brings about persistence of the urogenital sinus. On rectal examination, Dr. TeLinde felt a small uterus and a small ovary on each side. No tumor was felt in the pelvis nor could any mass be felt in the abdomen or kidney region. Intravenous pyelograms were negative. Blood counts, blood chemical studies, glucose tolerance, insulin sensitivity tests and a water excretion test for adrenal function were all negative. The 17-ketosteroid excretion varied between 19 and 22 mg. per day, of which less than 1 per cent was the beta fraction.

Embryonic hyperplasia of the adrenal could be excluded in this case because of the normal development of the vagina and urethra. The sudden and rapidly progressive onset of virilization at this age suggested that the child had a virilizing tumor of the adrenal gland or of some aberrant adrenal tissue. On December 31, 1943, Dr. Richard W. TeLinde and Dr. Alfred Blalock performed a transperitoneal exploration through which they were able to inspect the pelvis as well as the abdomen. Normal uterus, tubes and ovaries of average size for her age were found. Exposure of the right adrenal showed a tumor of dark brown color the size of a hen's egg attached to the lower pole of the

gland by a comparatively narrow pedicle (Fig. 6). It was removed leaving the upper half of the gland. The left adrenal appeared entirely normal.

Pathological Examination: "The adrenal tumor measures 4 × 3 cm. There is a capsule about 0.5 cm. thick. The lower two-thirds is rather firm and lobulated. The proximal third is cystic in consistency. At one point the capsule has been ruptured and protruding

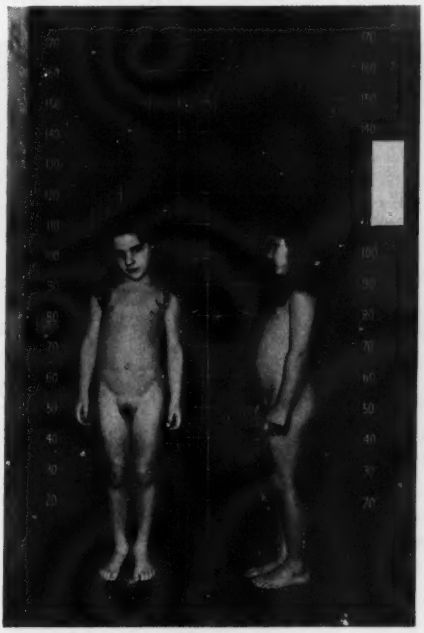

FIG. 5. *Case 2:* J.C. age 5½ years. Marked development of pubic hair. The greatly hypertrophied clitoris is not visible in the photograph. The vagina and urethra were normal.

through the opening is some very friable adrenal tissue with the typical brownish color. The tumor was attached by a pedicle about 1 cm. in diameter to the lower pole of the adrenal gland, which was not removed. The blood supply through this pedicle enters a small area of typical adrenal medulla.

Microscopic Examination: (Fig. 7) Sections show a well-encapsulated tumor composed of an irregular growth of cells having a granular pink-staining cytoplasm and containing nuclei which range in size from about 7 to 30 microns. The small nuclei have a dark pyknotic stain somewhat like lymphocytes. The larger ones have a reticulum and a very dark blue-staining central nucleolus. There are no mitotic figures, but there are a number of other pyknotic cells. There are scattered areas of soft calcification from 3 to 40 microns in diameter. In one of the sections there is a small amount of adrenal tissue outside the capsule. This is composed of strands of the usual small cells. Except

for the intact capsule and the absence of mitotic figures, the great disparity of size in the cell structure and of the staining qualities of the nuclei suggest a malignancy."

Following operation the child suffered no shock and recovered rapidly. The pubic hair which had been shaven grew rapidly again for about 3 weeks but was more scant than before operation. Her seborrhea and acne decreased. The 17-ketosteroid excretion which was from 19 to 22 mg. before operation decreased to 2.8 mg. the third day after operation, and to 1.4 mg. the eighth day. The patient was seen again in April, 1944. She had grown

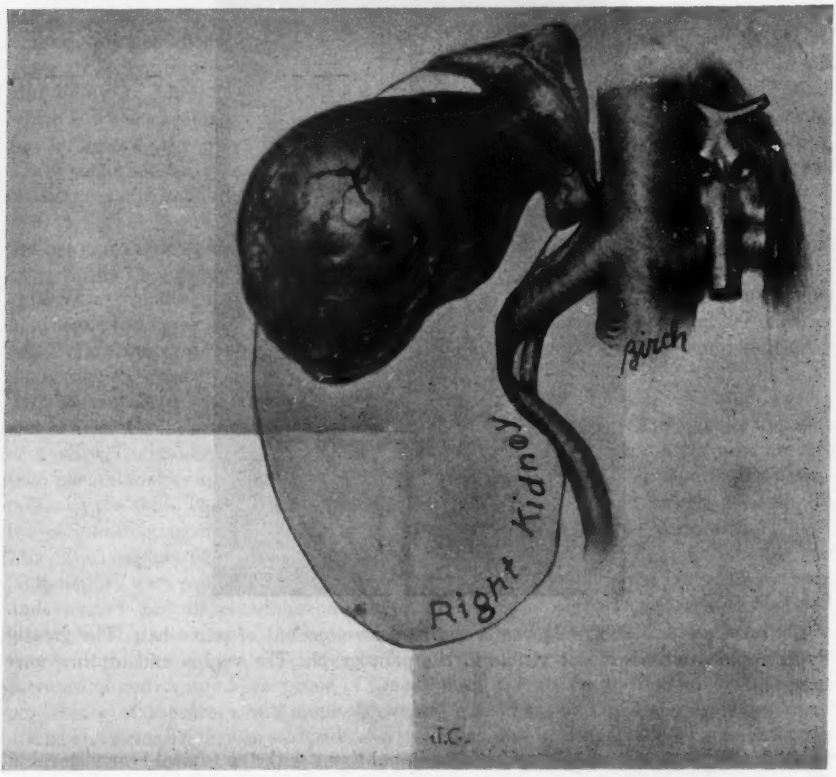

FIG. 6. *Case 2:* Drawing of adrenal tumor causing virilization.

2.2 inches since December, 1943. It was thought that the pubic hair was a little less dense than before operation and the clitoris was a little smaller. The excretion of 17-ketosteroids was 2.1 mg. per day. When she was seen again in December, 1944, the patient's height was 49.6 inches, representing a growth of 5 inches during the preceding year. The bone age, which was 7 the previous year, was now 11 years. The acne was more marked. Pubic hair was more abundant, a few hairs had appeared in the axillae and there was a little dark hair on the upper lip. Her voice was slightly deeper than previously. There was no change in the clitoris. There was no development of the breasts. The

vaginal smear indicated no estrinization. The 17-ketosteroids were 94 mg. per day December 27, and 126 mg. per day, December 28. X-rays of the chest and bones showed no metastasis. Intravenous pyelograms were negative. It was believed that the tumor had recurred or metastasized, and another abdominal exploration was undertaken by Dr. TeLinde on January 6, 1945. His note was as follows:

"On the lateral border of the ascending colon, about 2 inches from the tip of the caecum, there was a yellowish pedunculated tumor about the size of a golf ball. It had

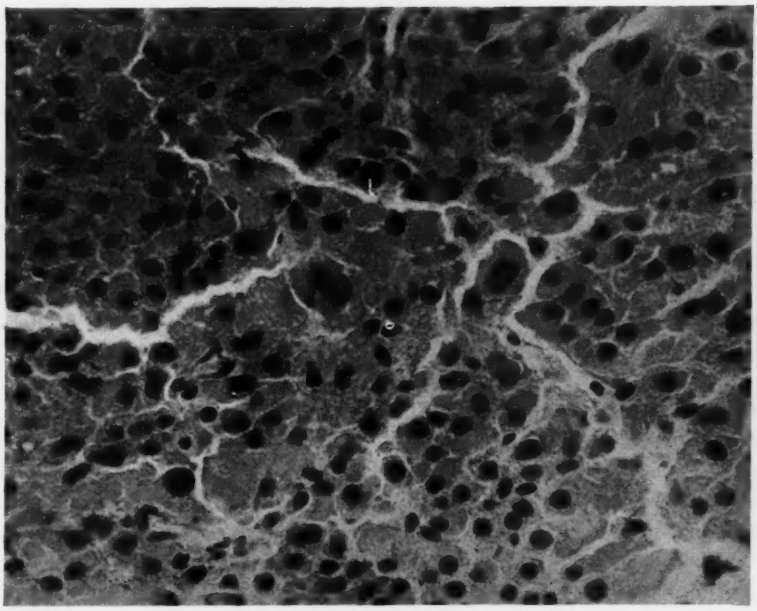

FIG. 7. *Case 2:* Microphotograph (×400) of adrenal tumor causing virilization.

the smooth appearance of the circumscribed tumor which was removed from the adrenal last year. At the hepatic flexure of the colon there was another similar tumor which was slightly larger. This tumor seemed quite free and very well encapsulated beneath the serosa of the colon. On the under surface of the right lobe of the liver to the right of the gall bladder a third tumor was present, which had the general appearance of the others but was more irregular in shape and blended with the liver substance. Above the upper pole of the right kidney in the region of the adrenal there was a small nodule about the size of a pea which probably represented a recurrence of the original tumor but there was no other evidence of recurrence in the adrenal region. The two large tumors attached to the colon were readily removed. The metastasis attached to the liver had apparently invaded that organ and it was impossible to remove it completely. The small tumor in the adrenal region was not removed."

Pathological Report: "The several sections of the three tumors show an identical microscopic picture. There are large sheets of the cells, the nuclei of which vary tre-

mendously in size and staining reaction. Mitotic figures are extremely rare. There is an abundant amount of pink cytoplasm and practically no connective tissue stroma. The tumor is well supplied with blood but in spite of this there are a few areas of necrosis and in these regions there is a mild infiltration with inflammatory cells. In the section taken from the liver there is invasion and destruction of the liver cells."

Following this operation there was a temporary decrease of the 17-ketosteroid excretion which then increased again as shown by the following:

17-Ketosteroids per day

12- 3-43	22.0 mg.
12- 4-43	19.0 mg.
12- 5-43	21.0 mg.

First operation 12-31-43

1- 3-44	2.8 mg.
1- 5-44	2.5 mg.
1- 6-44	2.4 mg.
1- 7-44	0.9 mg.
1- 8-44	1.4 mg.
1- 9-44	1.5 mg.
1-12-44	1.7 mg.
4- 8-44	2.1 mg.
12-27-44	94.0 mg.
12-28-44	126.0 mg.

Second operation 1-4-45

1-10-45	16.0 mg.
1-14-45	18.0 mg.
1-21-45	35.0 mg.
2- 1-45	38.0 mg.
3- 6-45	70.0 mg.
4-15-45	124.0 mg.

Following the second operation she returned to her home in another city. She is said to have remained strong and healthy until 2 weeks before her death in December, 1945. No details of her last illness are available.

DISCUSSION OF CASES

The hormonal effects of the two adrenal tumors were quite different. The girl showed enlargement of the phallus, precocious growth of sexual hair, seborrhea, deepened voice, somatic and muscular growth and accelerated epiphysial development. All of these symptoms can be attributed to excessive secretion of androgen. There were no signs of excessive cortin activity such as obesity, striae, plethora, hypertension or diabetic sugar curve. The lack of breast development and the absence of adolescent changes in the labia minora or vaginal epithelium indicated that estrogen was not being produced in as great amounts as in the adolescent period, or else that its effects were suppressed by excessive androgen. In the case

of the boy there were no evidences of excessive production of either androgen or cortin, and the excretion of 17-ketosteroids was low, not exceeding 4 mg. per day. The enlargement of the breasts suggested that estrin was being elaborated in excess. However, the breasts differed from normal female mammae in containing a large amount of connective stroma with little proliferation of ducts. Breast development cannot be considered proof of estrogenic activity because experimentally mammogenic effects may be produced by testosterone, chorionic gonadotropin or desoxycorticosterone. The cause of the breast enlargement in adolescent males is uncertain. In the syndrome described by Klinefelter et al. (2) and by Heller and Nelson (3), Albright has postulated that the gynecomastia is due to lack of "inhibin" secreted by the seminiferous tubules while androgen continues to be secreted by the interstitial cells. The fact that our patient had an enlarged prostate which seemed to be firm and fibrous suggested that there might be hypertrophy of the fibrous stroma, such as is caused by injecting estrogens into male animals. Further evidence that the patient was secreting an excess of a hormone which behaved like an estrogen was afforded by the fact that an extract of his urine caused cornification of the vaginal epithelium in a castrate rat in a titer slightly greater than ordinarily found in a preadolescent or even in an adult male.

Although in one case the hormone produced by the adrenal tumor behaved like an androgen, and in the other like an estrogen, no information was obtained concerning the chemical structure of the steroids in the tumor tissue. The actual androgenic content of hyperplastic or neoplastic adrenal glands is very low despite the fact that they may give rise to excessive amounts of androgenic steroids in the urine (Crooke and Callow (4), Slot (5)). In our cases no attempt was made to isolate and identify the steroids in the small amounts of tumor tissue obtained. However, biologic assays for androgen and estrogen were made by Dr. Harry Hays, of the Ciba Pharmaceutical Products, Inc. on acetone and acetone-benzene-ether extracts of the tumors with negative results.

The study of the steroids of the urine has yielded more information than the analysis of tissue extracts. Not only are there increased quantities of these substances in cases of adrenal tumor or hyperplasia, but in some instances large amounts of abnormal steroids not found in normal urine are excreted (Wintersteiner (6)). It has been suggested by Talbot (7) that the finding of a large proportion of the beta fraction of the 17-ketosteroids is diagnostic of adrenal tumor. However, an increased beta fraction is not invariably found in cases of adrenal tumor and there may at times be some increase with hyperplasia (Dobriner (8)). Frank (9) found very high excretions of estrogens in some cases of virilizing tumor and considered that this was characteristic of adrenal carcinoma rather than adenoma.

In our patient with virilizing tumor, urine collected prior to operation contained from 19 to 22 mg. of 17-ketosteroids per day; and after metastases occurred, as much as 124 mg. (Specimens were sent to Dr. Konrad Dobriner of Memorial Hospital Research Laboratories, New York City, for isolation of the steroids but as yet no report has been obtained.) The boy with gynecomastia excreted only 4 mg. of 17-ketosteroids and insufficient urine was collected before operation to permit proper isolation of individual steroids. In a later section in which we review cases of feminizing adrenal tumor in adult males, it will be shown that there may be considerable variation in the steroid excretion in this condition. Using bioassay methods on the urine, Simpson and Joll (10), and Roholm and Teilum (11) demonstrated a marked increase of estrogenic and only slight increase in androgenic activity. We shall refer later to an unpublished case of feminizing tumor studied by W. W. Scott in which large amounts of beta 17-ketosteroids and only small amounts of estrogen were found in the urine. The biologic effects of abnormal steroids of the adrenal, found in large amounts only in pathologic conditions, is not fully understood.

The pathologic study of the two tumors added little to our knowledge. In each of them the cells were large, polyhedral and showed no particular structural arrangement. The protoplasm was deep-staining and granular, and did not show vacuoles. In fact, Dr. Blackman was able to detect no essential differences in the two cases and considered that each neoplasm resembled the prenatal zone of the adrenal, and was probably derived from it. The Broster-Vines (12) stain for fuchsinophilic granules did not aid in differentiation, as these granules were abundant in both cases. This differed from the experience of Simpson and Joll (10) who studied a malignant adrenal tumor causing gynecomastia in a male and compared it with a virilizing adrenal carcinoma in a woman. They reported that a different type of cell was found in the two tumors; and the virilizing tumor showed a "diffusely fuchsinophilic tinge." However, Sudds (13) concluded that the sex hormones play no part in determining the presence of fuchsinophilic granules in the adrenal. Blackman (1) came to the conclusion that all cells which stain intensely with eosin such as liver cells and the reticular zone cells of the adrenal, show granulation with the ponceau-fuchsin stain, whereas cells filled with lipoids are not stained.

CLASSIFICATION OF ADRENAL DISORDERS

The hormones of the adrenal cortex, their biologic effects and relations to pathologic conditions have been extensively discussed (6, 12, 14, 15, 16, 17, 18, 19, 20, 21). They fall into five groups of steroids differing in chemical structure and biologic activity: 1) corticosterones having no

oxygen attached to C_{11} which are concerned principally with the control of the electrolyte balance; 2) corticosterones with an atom of oxygen on C_{11} which influence the carbohydrate metabolism; 3) androgens; 4) estrogens and 5) progestins. Adrenal tumors may exhibit no hormonal manifestations or may cause symptoms attributable to the excessive production of one or of a number of different hormones (Cahill (18)). Excess of the glycogenetic cortins which tend to cause the utilization of amino acids for

TABLE 1. CLASSIFICATION OF DISEASES OF ADRENAL CORTEX ACCORDING TO HORMONAL MANIFESTATIONS

	Pathological Etiology	Hormonal Dysfunction Cortin	Androgen	Estrin
Addison's Disease	Adrenal TBC Adrenal Atrophy Pituitary Deficiency	−	−	
Cushing's Syndrome	Hypothalamic? Pit. Basoph. Tumor Adrenal Hyperplasia Adrenal Tumor	+++	+	− or ±
Adrenogenital Syndrome Female—Pseudohermaphroditism Male—Macrogenitosomia Precox	Embryonic Hyperplasia	N or − − (Frequent)	++ ++	
Virilization	Postnatal: Tumor Postnatal: Hyperplasia	N	+++ ++	
Feminization—Gynecomastia	Feminizing Tumor			++

glycogen formation and to inhibit protein anabolism, may account for the diabetic sugar curve, obesity, evidences of protein deprivation and other signs of metabolic disturbance encountered in Cushing's syndrome (Albright (19)). Occasional disturbances of the electrolyte balance and perhaps hypertension may result from excess production of adrenal cortins (McQuarrie (20, 21), Anderson and Haymaker (22)). Excessive elaboration of androgen leads to increased growth and muscular development, hirsutism, acne and virilization.

In view of the multiplicity of the hormones which may be elaborated, it is not surprising that adrenal disorders may cause a great variety of symptoms, or that there may be gradations between the adrenogenital syndrome and Cushing's syndrome. It would avoid confusion to define the *adrenogenital syndrome* as a disorder in which there are evidences of excessive production of adrenal androgen with either (a) no evidence of disturbed

TABLE 2. ADRENAL TUMORS IN CHILDREN 12 YEARS OR YOUNGER, CLASSIFIED ACCORDING TO HORMONAL MANIFESTATIONS

	Total Cases	Adrenogenital Syndrome	Cushing's Syndrome				Feminization with Gynecomastia	Proven Malignancy	Operative Results		
			Obesity and Hirsutism	Obesity and Hirsutism with Early Female Development	Obesity only	Total			Exploration Only	Operative Removal	Cured
Female	53	31*	14	6	2	22		21	4	28	11
Male	17	12	2		2	4	1	9	2	7	3
Total	70	43	16	6†	4‡	26	1	30	6	35	14

* Three cases were adrenal tumors of the ovary—Riche (36), Gaudier (35), Downes and Knox (34).

† Precocious breast development and/or menstruation in cases of Tilesius (37), Bullock and Sequeira (38), Walters, Wilder and Kepler (39), Friedgood and Gargill (30) and in two cases of ovarian adrenal tumor, Riche (36) and Gaudier (35).

‡ Females showing obesity without hirsutism were Gross's patients 4 and 5 (29)—both cases of short duration. Males were Lightwood's patient aged 4½ months (32); and Taylor and Wiseman's (40) aged 11 months, who had a large penis.

cortin production or (b) deficiency of cortins:[1] and *Cushing's syndrome* as a disorder in which signs of excessive cortin production predominate, with usually some evidence of increased androgen and occasionally increased estrin. On the basis of these definitions the classification of adrenal disorders is shown in Table 1.

ADRENAL TUMORS IN CHILDHOOD

Cases of adrenal tumor in children have been collected from the literature by Reilly, Lisser and Hinman (26)—37 cases; Marks, Thomas and Warkany (27)—24 cases; Wilkins, Fleischmann and Howard (23)—11 cases in the male; and Goldstein, Rubin and Askin (28)—54 cases. Additional cases not included in these series have been reported by Gross (29), McQuarrie (20), Friedgood and Gargill (30), Pratt and Schaefer (31), Lightwood (32) and Wharton (33). Actually, excluding all duplications and adding our 2 cases, there are 70 children (53 girls and 17 boys) who developed adrenal tumor before the age of 12 years. In three of these cases, the neoplasm occurred in aberrant adrenal tissue in or near the ovaries (34, 35, 36). We have reviewed the published abstracts of these histories to determine which patients showed marked obesity, plethora, hypertension or other evidences of excessive cortin secretion, and which showed virilization with accelerated growth and muscular development suggesting only hypersecretion of androgen. The findings are shown in Table 2.

Of the 53 girls, 31 fulfilled our definition of simple adrenogenital syndrome, and 22 had symptoms suggestive of Cushing's syndrome. Of the 22 girls who were obese or had other manifestations of hypercorticism, all but 2 (29) who ran an unusually rapid course showed hirsutism or other evidence of excessive androgen, and 6 gave evidence of precocious secretion of estrin.[2]

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[1] The fact that embryonic hyperplasia of the adrenal causing macrogenitosomia praecox or female pseudohermaphroditism may lead to cortico-adrenal insufficiency has been discussed by Wilkins, Fleischmann and Howard (23), Bratrud and Thompson (24) and Jaudon (25).

[2] A 4-year old patient of Tilesius (37) was excessively fat and had large mammae and well developed pubic hair.

A patient of Bulloch and Sequeira (38) was obese and hirsute, had large breasts and menstruated at 10 years.

Walters, Wilder and Kepler's (39) patient developed breasts at 4 years. Her menses started at 8 years and recurred every 2 months. In addition, she showed marked hirsutism, acne, deep voice, advanced bone age, obesity and hypertension.

Friedgood and Gargill's patient (30) menstruated at 8 years, had evidences of marked virilization and also had plethora, striae and hypertension.

In addition, Riche (36) and Gaudier (35) each reported an adrenal rest tumor of the ovary causing menorrhagia and metrorrhagia at about 4 years.

It is of considerable interest that although evidences of virilization were manifested in 12 cases during the first year of life, and in a number of these within the first few months, abnormalities in the differentiation of the genital tract with persistence of a urogenital sinus did not occur in any case.[3] This is in marked contrast to congenital adrenal hyperplasia which in females practically always causes pseudohermaphroditism with a persistent urogenital sinus.

Of 17 males with adrenal tumor (20, 29, 32, 40, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55), 12 showed precocious sexual development, accelerated growth and osseous development and were muscular but not obese. Only 2 showed obesity of the Cushing's type (32, 40). Our patient (Donald G.) is the only case of feminization with gynecomastia in this age group. It should be noted that when precocious development of the male genitalia and secondary sex characteristics result from androgen derived from adrenal neoplasm or hyperplasia, the testes usually do not undergo adolescent development. In 6 cases the testes were described as small or normal, and in 7 cases they were not mentioned. Taylor and Wiseman (40) stated that in their 11-month old patient the testes were "larger than normal." In a 2½-year old patient of Fordyce and Evans (46), and a 3-year old boy of Macera (51) they were "large" but showed no sperm. In a 5-year old boy reported by Player and Lisser (52) the testes were said to be of adult size and erections and nocturnal emissions were reported. It is questionable whether spermatozoa were present. This is in marked contrast to the cases of males having sexual precocity due to brain lesions or to idiopathic premature activation of the pituitary, who usually develop mature adult testes with spermatogenesis.

The tendency of adrenal tumors to malignancy is shown by the fact that metastases occurred in 30 of the 70 cases. There is a high degree of operative mortality. Only 14 patients (11 girls and 3 boys) of the 35 in whom removal of the tumor was attempted were cured. The first successful operation was

[3] Soffer (14) quotes incorrectly the cases of Scabell (41) and Krabbe (42) as instances of female pseudohermaphroditism associated with adrenal tumor. Actually, Scabell's first case was one of adrenal tumor causing virilization without genital maldevelopment and his second case was a female pseudohermaphrodite with congenital adrenal hyperplasia. Krabbe did not report any cases. He emphasized that adrenal lesions do not cause true sexual precocity but heterosexual development which he termed "pseudohermaphroditism." DaRocha (43) incorrectly applied the term "pseudohermaphroditism" to a case of virilization due to adrenal tumor in a 2-year old child. The case has been quoted as having the vagina "obliterated." Actually, there was a normal vagina obscured by a large clitoris. Bratrud and Thompson's (24) *case 7* was a female pseudohermaphrodite of 32 years who had bilateral congenital adrenal hyperplasia and also a carcinoma of the right adrenal which might have developed subsequently.

performed by Collett in 1924. A number of the patients died from hemorrhage and it is probable that postoperative shock and death were due in some cases to the fact that the contralateral adrenal may have undergone atrophy due to the excessive hormonal activity of the tumor so that adrenocortical insufficiency followed the removal of the neoplastic gland. It is probable that with improved technic and supportive adrenal therapy a higher percentage of cures will be obtained in the future.

FEMINIZING ADRENAL TUMORS CAUSING GYNECOMASTIA IN ADULT MALES

Ten previously reported cases of gynecomastia (10, 11, 56, 57, 58, 59, 60, 61) due to adrenal carcinoma in adults are listed in Table 3. We are grateful to Dr. W. W. Scott, Professor of Urology, Johns Hopkins Hospital, for his permission to add an eleventh case, hitherto unpublished, which he studied in 1945 in Chicago. All of these patients were between 26 and 44 years of age with the exception of the 15-year old patient of Holl (57). Our patient (D. G.) is the only case in which a feminizing tumor of the adrenal has been found before the onset of puberty.

The high degree of malignancy is shown by the fact that 9 of the 12 patients died from metastases or postoperative recurrences. Holl's *patient 2* was reported as cured after operation. McFadzean's (59) patient was well six weeks postoperatively. Our patient shows no evidence of recurrence or metastases four years after operation.

The breasts and nipples were well developed in all cases. Increased pigmentation of the nipples was noted in the two cases of Holl (57). In the case of zum Busch (61) a little milky fluid could be expressed, and in Lisser's case there was a watery secretion. All the patients apparently had some loss of libido and in 6 cases the testes were described as small or atrophic. In only two cases was there any tendency to obesity. Holl's 44-year old patient showed increased adiposity with the onset of symptoms, and lost weight following the successful removal of the tumor. The patient of Simpson and Joll (10) gained nearly 30 lbs. of weight during the first year after gynecomastia appeared, and then lost about 60 lbs. prior to operation. Following operation he regained his original weight but again lost when the tumor recurred. Hypertension or other evidences of Cushing's syndrome are not recorded in these cases. In Bittorf's (56) case there was increased pigmentation of the eyelids and face and in Holl's first case there was a pigmented linea fusca on the abdomen.

In only four cases have studies of the urinary hormones been made. In the cases of Simpson and Joll (10) and of Roholm and Teilum (11) bioassays showed only a slight increase of androgen but a large increase

TABLE 3. FEMINIZING ADRENAL TUMORS IN MALES

Author	Year	Age Yrs.	Breasts		Androgenic activity			Skin Pigment	Obesity	Androgen Bioassay	Urinary Assays		Outcome
			Enlargement	Secretion	Reduced Libido	Atrophy of Testes	Decreased Sexual Hair				Estrogen Bioassay	17-Ketosteroids	
1. Bittorf (56)	1919	26	+	0	+	+	0	+	0				Died—metastases
2. zumBusch (61)	1926	27	+	Milky					0				Died—metastases
3. Holl (57)—*case 1*	1930	15	+	0		0	+	+	0				Exploration—inoperable—died
4. Holl—*case 2*	1930	44	+	0	+	+	+	0	+				Operation—well
5. Lisser (58)	1936	33	+	Watery			0		0				Died—metastases
6. Simpson & Joll (10)	1938	34	+	0	+	+	+		+	50–100 c.c.u† per liter	3000 m.u./l		Operation—recurred—died
7. Pico Estrada (60) *Case 1*	1940	30	+	0		+	0	+	0				Died—metastases
8. Pico Estrada—*case 2*	1940	41	+	0			?	0	0				Died—metastases
9. Roholm and Teilum (11)	1942	44	+	0	+	+	+	0	0	5–80 c.c.u./24 hrs.	5000 m.u./24 hrs.		Died—metastases
10. McFadzean (59)	1946	29	+	0	+	0	0	0	0				Operation—well six weeks later
11. W. W. Scott		43	+	0	+	+	0	0	0		5.3 m.u./24 hrs. and estradiol	196 mg./24 hrs.	Operation—metastases—died
12. Wilkins		5	+	0		0		0	0		5 R.U./24 hours‡	4 mg./24 hrs.	Operation—well after four years

† c.c.u. = capon comb units. m.u. = mouse units.
‡ R.U. = rat units

of estrogen (5000 mouse units per day compared to less than 20 mouse units in normal individuals). W. W. Scott made extensive chemical studies of the steroids excreted by his patient. The total 17-ketosteroids amounted to 195.6 mg. per day. Of 189.8 mg. contained in the ketonic fraction there were only 5.8 mg. of alpha-ketosteroid while 70 mg. of crystalline dehydroisoandrosterone, a beta compound, were isolated. Estrogenic activity was equivalent to only 5.3 micrograms of alpha-estradiol benzoate per 24 hours. Following operative removal of the tumor the 17-ketosteroids decreased to 9.6 mg. per 24 hours with a reversal of the alpha-beta ratio. However, estrogenic activity was not decreased, being equivalent to 9.5 micrograms of alpha-estradiol benzoate per 24 hours. Analysis of the tumor showed no 17-ketosteroids although estrogens were found (data concerning the exact amount are not available). In our patient the excretion of 4 mg. of 17-ketosteroids per 24 hours was slightly greater than usually found at the the age of 5 years, but less than that of a normal adult. The estrogenic activity of 5 rat units per day represented only a questionable slight increase for a boy his age.

SUMMARY

A 5-year-old boy with marked gynecomastia was found to have an encapsulated adenoma of the adrenal cortex. After removal there was a gradual decrease in the size of the breasts and no evidence of recurrence after four years.

A 5½-year-old girl who had shown rapid virilization over a period of six months was found to have an encapsulated tumor of the adrenal cortex. After operation metastases recurred in the abdomen and the patient died two years later.

The feminizing tumor and the masculinizing tumor could not be differentiated histologically. In the case of gynecomastia the excretion of 17-ketosteroids was 4 mg. per day and small amounts of estrogen were demonstrated. The patient with virilization excreted 22 mg. of 17-ketosteroids before operation and 124 mg. after the tumor recurred. Less than 1 per cent consisted of the beta fraction.

Seventy cases of cortical adrenal tumor occurring in children under 12 years of age have been reviewed and classified according to whether they presented symptoms attributable only to excessive androgen or whether they also showed obesity and other metabolic disturbances suggestive of excessive production of cortin.

Ten cases of adrenal tumor causing gynecomastia in adult males are reviewed and an unpublished case with extensive hormonal studies by Dr. W. W. Scott is added. Our patient is the only instance of a feminizing adrenal tumor in a child.

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THE EFFECT OF STARVATION ON URINARY 17-KETOSTEROID EXCRETION*

RICHARD L. LANDAU, M.D., KATHRYN KNOWLTON, Ph.D., DOLORES ANDERSON, B.S., MINNIE B. BRANDT, A.B., AND ALLAN T. KENYON, M.D.

From the Department of Medicine of the University of Chicago

CHRONIC illness associated with malnutrition, debility and evidence of infection often leads to depression of urinary 17-ketosteroid values (1). Forbes et al. (2) report a rise in 17-ketosteroids following operations, fever or the sudden onset of an acute illness, followed by a decline after 12 to 48 hours which often reached subnormal levels. We have confirmed this in an unpublished study in a subject with induced fever in whom food intake was maintained unchanged. The numerous evidences, however, from studies on rats (3, 4) and dogs (5) that androgen production by the testes is reduced during inanition suggests that undernutrition alone may often contribute to the depression of 17-ketosteroid excretion in human disease. Accordingly the effects of starvation in three normal men and one obese woman were examined, together with those of relatively mild protein and caloric restriction in two normal men.

METHODS

The procedure differed somewhat among the subjects. The woman (H.F., age 30) and two of the normal men (R. L., age 27 and R. W., age 25) were on a constant, adequate, self-selected diet prior to starvation. One normal man (D. P., age 26) ate as he chose from day to day. Starvation in all instances lasted four days. R. W. was given 7 Gm. of salt daily in addition to water during this period. D. P. likewise received salt supplements; the others water only. The fast was terminated in all but R. W. by prompt resumption of the control diet. In R. W. four days of chiefly carbohydrate feedings (carb. 200 Gm., prot. 3 Gm., fat 0.6 Gm. daily) preceded resumption of the original diet. The men carried on their usual sedentary occupations throughout; the woman, although hospitalized, was up and about most of the time.

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In the protein depletion studies R. L. was shifted from an adequate control diet (carb. 282, prot. 108 (N 17.6), fat 158, cal. 2982) to a low protein diet with some caloric restriction (carb. 308, prot. 6.6 (N 1.1), fat 55, cal. 1755) for eight days and then returned to the original diet. A. K. (age 43) shifted from the control (carb. 285, prot. 86 (N 13.5), fat 148, cal. 2861) through two increasing grades of protein and caloric restriction before resuming the original diet. These were respectively carb. 343, prot. 29 (N 4.6), fat 85, cal. 2253 for six days and carb. 295, prot. 10 (N 1.7), fat 41, cal. 1589 for four days. The usual sedentary occupations were maintained.

In all subjects the 24-hour urine collections were checked for completeness by creatinine determinations (6). Urinary nitrogen (7) was determined daily except in D. P. Analyses for stool nitrogen were not made and the estimated nitrogen losses given are from urine only.

Total neutral 17-ketosteroids were determined daily. To confirm the course of the curves of excretion, 17-ketosteroids were measured in the ketonic fractions in numerous instances. Five hundred cc. aliquots of urine were hydrolyzed by boiling with 50 cc. of concentrated hydrochloric acid for 15 minutes, and then extracted with carbon tetrachloride for 2 hours in the extractor described by Consolazio and Talbott (8). The Zimmermann reaction was carried out by the method of Holtorff and Koch (9) in which aqueous potassium hydroxide is used. Androsterone served as a standard, except with H. F., when it was unavailable. Here dehydro-iso-androsterone acetate was used and the values were expressed in terms of dehydro-iso-androsterone. The color was allowed to develop for 45 minutes. Color corrections for non-ketonic chromogens were not made. Engstrom and Mason(10) have shown them to be inaccurate when applied in the Holtorff-Koch procedure.

For ketonic fractions, which are useful with the Holtorff-Koch procedure (10), separation from the crude material was accomplished by reaction with Girard's reagent T (11).

Since practice still differs considerably in detail among several laboratories measuring 17-ketosteroids, and since precise specificity is hardly claimed by any, the figures given have chiefly relative significance for the experiments at hand and will deviate somewhat in absolute amounts from those obtained by other methods elsewhere. The necessary change from androsterone to dehydro-iso-androsterone acetate as a standard in our own work presumably made some difference in the absolute values obtained (12).[1]

[1] Androsterone and dehydro-iso-androsterone acetate were generously supplied by Dr. Irwin Schwenk of the Schering Corporation.

The androgenic activity of certain specimens was determined by measuring the growth of the capon's comb after local application of the neutral extract taken up in ether according to the method of Johnston and Koch (13). Ten birds were used in each assay. Androsterone served as the standard and the results are expressed in International Units (0.1 mg. androsterone per unit). This method, which is unpublished, may be considered roughly comparable to the injection method of Gallagher and Koch (14). As used here the data may be considered approximate only.

RESULTS

Fasting induced a progressive decline in the excretion of 17-ketosteroids in all 4 experiments (Figs. 1–4). This was always well defined by the third day and reached approximately 50 per cent of the baseline values by the fourth day. Spot determinations of the ketonic fractions paralleled those

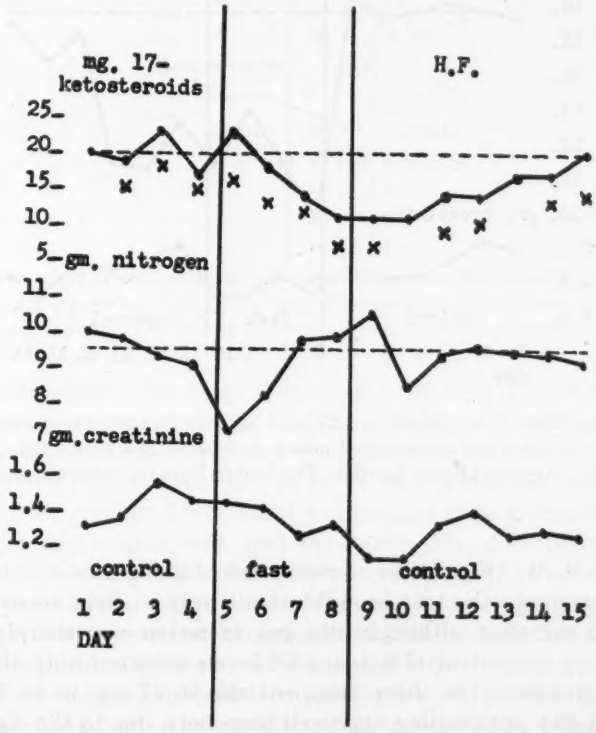

FIG. 1. The effect of starvation in a 30 year old woman on the urinary excretion of 17-ketosteroids, nitrogen and creatinine. Control diet: carb. 214, prot. 71 (N 11.35), fat 97, cal. 2013. x signifies ketonic fraction. The broken lines represent the average of control values.

of total neutral 17-ketosteroids. Supplements of salt during the fast made no difference in the results.

The course during recovery varied somewhat, depending upon experimental events. In H. F. and R. L. (Figs. 1, 2) 17-ketosteroids rose slowly and regularly to the control levels after 7 and 4 days respectively of normal

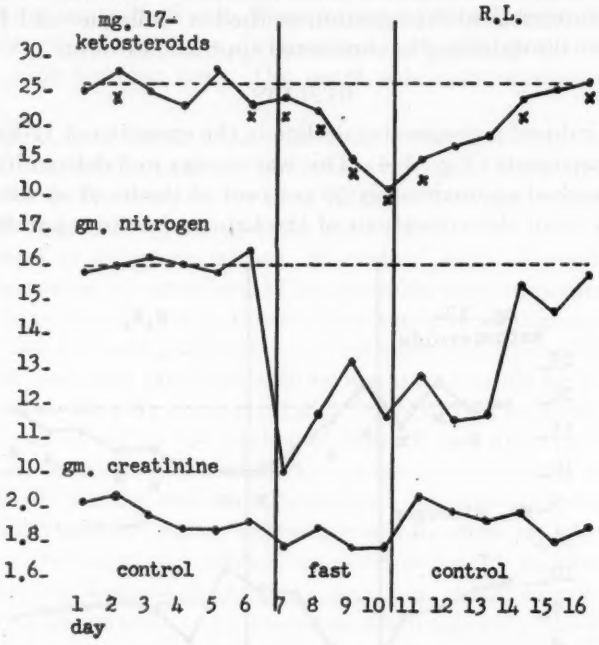

FIG. 2. The effect of starvation in a 27 year old man on the urinary excretion of 17-ketosteroids, nitrogen and creatinine. Control diet: carb. 255, prot. 121 (N 19.3), fat 173, cal. 3061. x signifies ketonic fraction. The broken lines represent the average of control values.

feeding. In R. W. (Fig. 4) the consumption of 200 grams of carbohydrate daily to terminate the fast brought about only a slight recovery of 17-ketosteroid excretion although nitrogen excretion was sharply reduced. Full recovery to control 17-ketosteroid levels occurred only after 9 days of the normal diet. The sharp transient rise to 27 mg. in D. P. (Fig. 3) on the first day of refeeding may well have been due to the diodrast and insulin clearances, including painful catheterization, that were done as part of another study. Pincus' review (15) may be consulted for an account of the extensive work of his own group on the stimulating effects of psycho-

motor activity on 17-ketosteroid excretion. The discomfort and reaction in D. P. may well be analogous to certain elements in Pincus' experiments. Indeed, it is perhaps surprising that a phenomenon as labile as 17-ketosteroid excretion often seems to be, should prove so stable under the ordinary working conditions of our control studies.

The androgenic activity of selected samples of urine paralleled the 17-ketosteroid excretion in the three subjects studied. Since these data are not charted, they will be recorded briefly here. In R. L. the androgens fell from a prefasting level of 95 I. U. per day to 40 I. U. per day on the third and fourth days of the fast. Assays on the first and fourth days of refeeding

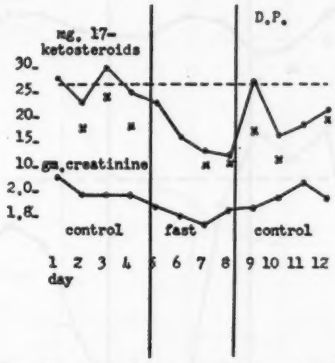

FIG. 3. The effect of starvation in a 26 year old man on the urinary excretion of 17-ketosteroids and creatinine. During the control periods the subject was on a free diet. x signifies ketonic fraction. The broken line represents the average of control values.

provided estimates in the neighborhood of 30 to 40 I. U. per day. On the sixth day of recovery a value of 75 I. U. per day was reached. In D. P. the initial value of 45 I. U. per day was reduced to 25 I. U. per day on the third and fourth days of the fast. In R. W. two prefasting estimates were 55 and 85 I. U. per day. Days 3 and 4 of the fast gave estimates of 30 and 15 I. U. per day respectively, and the fourth day of carbohydrate diet, only 30 I. U. per day. On the ninth day of refeeding an excretion of 50 I. U. per day was estimated. Since most of the androgenicity of normal urine is attributable to androsterone, these findings indicate a decline in the excretion of this steroid roughly paralleling that of total 17-ketosteroids.

The moderate nitrogen depletion produced in A. K. and R. L. by the low protein diets, somewhat restricted in caloric value (Figs. 5, 6), did not alter 17-ketosteroid excretion. Although the amount of nitrogen lost (A. K. 34 Gm. in 10 days; R. L. 43 Gm. in 8 days) approximated that lost by the fasting subjects (R. L. 46 Gm., R. W. 43 Gm., H. F. 35 Gm.,

each in 4 days) the physiological significance of the two types of nitrogen depletion may well differ sufficiently to mar comparison. In any event no evidence is adduced for any special sensitivity of the forces governing 17-ketosteroid production and excretion, to substantial reduction in protein intake.

Our attempt to measure testicular function indirectly during fasting by

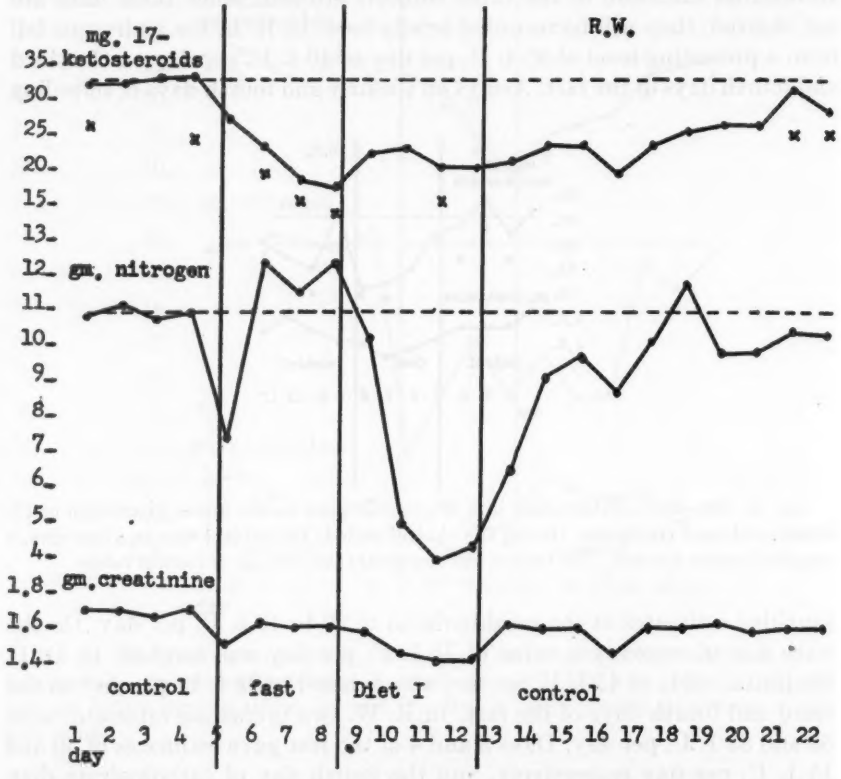

FIG. 4. The effect of starvation in a 25 year old man on the urinary excretion of 17-ketosteroids, nitrogen and creatinine. Control diet: carb. 308, prot. 84 (N 13.4), fat 125, cal. 2693. Diet I: carb. 200, prot. 3, fat 0.6, cal. 821. x signifies ketonic fraction. The broken lines represent the average of control values.

determining the urinary acid phosphatase excretion may be mentioned briefly, chiefly for the purpose of pointing up the necessity of developing corroborative measurements of testicular function when 17-ketosteroids are followed in the normal male. This enzyme is contributed in substantial part by the prostate and is excreted in the urine with some degree of con-

stancy in a given individual when discharge of seminal fluid is avoided. This phenomenon is indicated by a sharp peak in excretion which greatly exceeds the values usually obtained. In R. W. five control values ranged from 360 to 1050 (King Armstrong) units (avg. 592) per day. Values during the four days of fast ranged from 304 to 724 units (avg. 443); during the four days of carbohydrate feeding from 570 to 612 units (avg. 594);

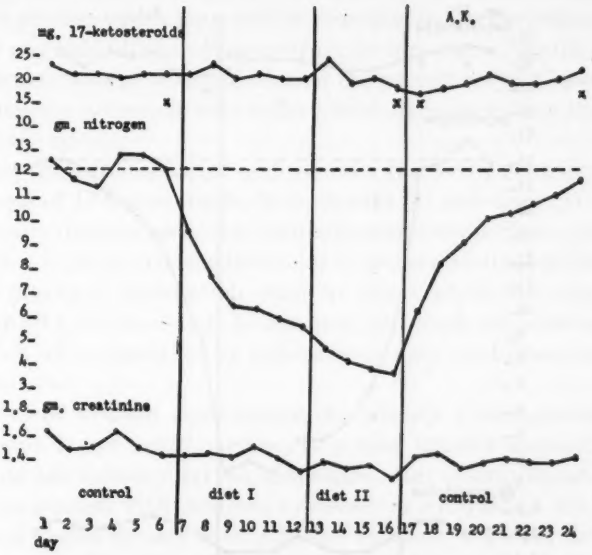

FIG. 5. The effect of low protein diets in a 43 year old man on the urinary excretion of 17-ketosteroids, nitrogen and creatinine. Control diet: carb. 285, prot. 86 (N 13.5), fat 148, cal. 2861. Diet I: carb. 343, prot. 29 (N 4.6), fat 85, cal. 2253. Diet II: carb. 295, prot. 10 (N 1.6), fat 41, cal. 1589. x signifies ketonic fraction. The broken line represents the average of control values.

and during the ten days after return to the original diet, from 320 to 712 (avg. 446), with no evidence of progressive change. In D. P. four baseline values ranged from 864 to 1159 units (avg. 1030); four during fasting, from 634 to 828 (avg. 748); and three during recovery, (excluding one of 3815, due presumably to seminal discharge) from 366 to 568 (avg. 491), with no progressive change. Little evidence of depressed testicular function during fasting can be extracted from such data. We are ignorant however of the time required for given degrees of testicular impairment to be reflected in prostatic involution, as represented by declining urinary phosphatase levels. It is thus by no means certain that our fasts were long enough to indicate diminished androgen production measured in this way.

COMMENTS

A consistent decline in the urinary excretion of total neutral 17-ketosteroids, confirmed by determinations of 17-ketosteroids in the ketonic fraction after Girard separation, was clearly defined by the third day of fasting in the three men and one woman studied. Estimations by biological assay indicated a parallel influence on the androgenic constituents. Such

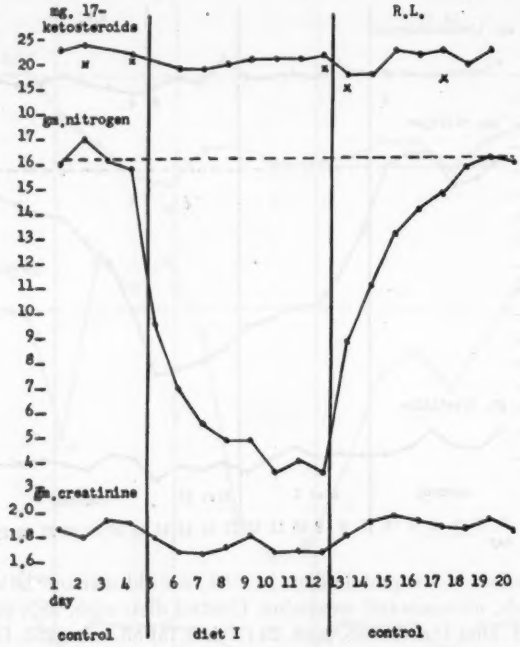

FIG. 6. The effect of a low protein diet in a 27 year old man on the urinary excretion of 17-ketosteroids, nitrogen and creatinine. Control diet: carb. 282, prot. 108 (N 17.6), fat 158, cal. 2982. Diet I: carb. 308, prot. 6.6 (N 1.1), fat 55, cal. 1755. x signifies ketonic fraction. The broken line represents the average of control values.

changes may be produced by any process which influences the rate of secretion of hormonal or other precursors concerned; the utilization of such hormones by receptive target cells; or the decomposition of such hormones and the conjugation and excretion of their resulting derivatives. Interpretations must accordingly be qualified by the uncertainties surrounding these events.

It is unlikely that the withdrawal of immediate dietary precursors is responsible. The low 17-ketosteroids in those patients with hypopituitarism and in women with Addison's disease who are eating normally or nearly so,

demonstrates that food does not inevitably supply 17-ketosteroids or their precursors. The possibility that food or a physiological substance such as bile, active in the handling of food, may contribute 17-ketosteroids only under the mediation of pituitary or adrenal secretions is without supporting evidence. Sharp reduction in protein intake did not induce the characteristic changes of starvation.

Inanition interferes with the inactivation of secreted estrogens by the liver (16), a process which may well be essential to the derivation and conjugation of steroids ultimately appearing in the urine. Should starvation similarly impair hepatic manipulation of the precursors of 17-ketosteroids, declining urinary excretion may reflect liver damage rather than altered secretion of hormones.

The testis, the adrenal cortex and possibly the ovary contribute hormonal precursors of 17-ketosteroids. In both rats (4) and dogs (5) starvation does eventually depress testicular androgen production. Since reactivity to gonadotropins is preserved, the testicular defect is presumably secondary to diminished pituitary stimulation. Such an effect would account very well for the reduced 17-ketosteroids in our men, although our attempts to corroborate such an explanation by following urinary acid phosphatase were hardly definitive.

The extent of ovarian contribution to urinary 17-ketosteroids in the normal woman is not entirely clear. It is now usually assumed that the relatively normal values observed some time after castration and the very low values in women with Addison's disease, in contrast to the less strikingly reduced values in men with Addison's disease, together demonstrate that the ovary, unlike the testis, makes no such contribution. Such considerations suggest that if the parallel decline in urinary 17-ketosteroid excretion during starvation in the two sexes is to have a common explanation it should be sought either in adrenal defect or in peculiarities of hepatic function rather than in the gonads. The experience of Scott and Vermeulen (17), however, deters us from pressing this point too firmly. In older men castration for prostatic cancer induced only a temporary decline in urinary 17-ketosteroid excretion. Until such carefully constructed experiments are available on women, we hesitate to use normal values some time after castration as fairly representing all phases of the response to loss of ovarian secretion. Our four day starvation, of course, would correspond in time to the early days after castration. To our minds for the moment, ovarian defect arising from starvation is not certainly excluded from responsibility for the declining urinary 17-ketosteroids of our woman.

It is possible in these experiments that depression of adrenal cortical secretion has occurred during starvation. In fasting rats hypertrophy of the adrenal cortex has been demonstrated (18), and interpreted

as meaning the participation of adrenal agents in the mobilization of glucose from tissue precursors (19). To the extent that such a process in man is reflected in urinary 17-ketosteroid excretion, one would expect an increase rather than a decrease during starvation. Many uncertainties, however, surround our understanding of the nature of the adrenal precursors of 17-ketosteroids in man. During starvation one would expect the enhanced secretion of a glucose-mobilizing, glycogen-depositing, nitrogen-expelling substance similar to corticosterone or its near relatives. Only recently Thorn (20) and Mason (21) and their associates have demonstrated rising urinary 17-ketosteroids after the administration of adrenotropins to man. In the experience of the Boston group the physiological effects were reminiscent of the properties of corticosterone or one of its relatives. The detailed studies of Venning and Browne (22), however, conducted largely after damaging events have not shown a strict agreement between the excretion of 17-ketosteroids and that of the clearly adrenocortical-like compounds of human urine. The suggestion (23, 24) that certain cortical precursors of normal 17-ketosteroids are anabolic and androgenic substances and hence unlike corticosterone or a near relative, should be kept in mind. To the extent that urinary 17-ketosteroid excretion reflects the activities of such a hypothetical agent, the physiological meaning in terms of nitrogen metabolism, for example, may be quite different from and indeed opposite to any reflection of the activity of a corticosterone-like compound.

Since possible precursors of 17-ketosteroids may exert either anabolic or catabolic influences on body protein, the relation between 17-ketosteroid excretion and the behavior of tissue protein in these experiments will be briefly summarized. No strict correlation between nitrogen excretion and 17-ketosteroid excretion appears from our data. Thus while in R. L. (Fig. 2) nitrogen excretion declined during fasting from the initial high values of a diet rich in protein somewhat in advance of the decline in 17-ketosteroid excretion, in R. W. (Fig. 4) urinary nitrogen rose above the control level as 17-ketosteroid excretion declined. Furthermore the termination of the fast by carbohydrate feedings in R. W. elevated 17-ketosteroids only slightly at a time when nitrogen excretion was sharply reduced and the rapid rise in nitrogen excretion on refeeding the original diet was followed only gradually by a rise in ketosteroids. In H. F. (Fig. 1) the later fasting values of urinary nitrogen corresponded with those of the control period, although 17-ketosteroid excretion was depressed. In A. K. and R. L. (Figs. 5, 6) the decline in nitrogen excretion on the low protein diets was unaccompanied by a distinct alteration in 17-ketosteroid excretion. Thus, although steroids with anabolic properties of a generalized character reduce urinary nitrogen excretion, no evidence from 17-ketosteroid excretion is

adduced here that they participate in any immediate and direct manner in those adjustments of the organism to variations in food consumption that are expressed by urinary nitrogen excretion. Nor do the variations in ketosteroid excretion fit the activities of a catabolic agent any better than they do those of a presumed anabolic agent. It is accordingly entirely likely that these variations are secondary and may be the result of pituitary inactivity induced by starvation, although they may represent processes that once expressed, are of some eventual metabolic value. It is possible, for instance, that any reduced anabolic force reflected in diminished 17-ketosteroid excretion during fasting would facilitate dissipation of tissue proteins by an active catabolic agent. Such a possibility is to be distinguished from immediate and direct responsibility for a process.

It is thus difficult to read a direct and simple interpretation of secretory phenomena from variations in 17-ketosteroid excretion in normal men and women subjected to starvation. In any event it is apparent that starvation is one factor in illness that will contribute to lowering urinary 17-ketosteroid values in either sex, and that restoration does not readily occur on small carbohydrate feedings only. On the other hand loss of nitrogen from the body induced by low protein diets with moderate caloric restriction has little effect on 17-ketosteroid excretion even when the amount lost approximates that due to starvation. It is possible that the rate of nitrogen discharge or the differing source and physiological significance of that discharged determines the difference in response of 17-ketosteroids. More extensive protein depletion might well, of course, provide a response not indicated in these brief experiments.

SUMMARY

1. In three normal men and in one obese woman starvation for four days produced a decrease of 50 per cent in the excretion of total neutral 17-ketosteroids and of those in the ketonic fraction after Girard separation. The effect was well defined by the third day. With restoration of a normal diet the values returned to their previous levels in about a week. In those studied (men), urinary androgen excretion was found to parallel that of 17-ketosteroids.

2. In two normal men no significant alteration in 17-ketosteroid excretion was produced by a diet low in protein and somewhat restricted in calories within the 8 to 10 day period of study.

3. More precise information concerning the source and metabolic pathways of the precursors of urinary 17-ketosteroids must be forthcoming before exact interpretation is possible.

4. Inanition is one factor that will cause a lowering of urinary 17-ketosteroid excretion in disease.

ACKNOWLEDGMENTS

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A COMPARISON OF ORAL AND VAGINAL EPITHELIAL SMEARS

DANIEL E. ZISKIN, D.D.S. AND RUTH MOULTON, M.D.

From the School of Dental and Oral Surgery and College of Physicians and Surgeons, Columbia University, New York

THIS study was undertaken for the purpose of comparing the relative merits of oral and vaginal epithelial smears in an attempt to simplify or supplement the clinical method of appraising the endocrine changes during the ovarian cycle in women. Vaginal smears have been shown to be useful in studying the menstrual cycle, and it was felt that similar changes might be reflected in the oral mucosa since the latter possesses certain features in common with the vaginal mucosa, when examined histologically. For example, both areas may simultaneously present signs of leukoplakia, lichen planus, ulcers, and other diseases. On the other hand, there are differences. The oral mucosa is composed of more layers of cells than the resting vaginal mucosa; the outer basal cell layer and the intermediate and superficial cell layers of the vagina do not have exact counterparts in the oral epithelium; the cornified cells, when present, comprise the most superficial layer in the oral epithelium while in the vaginal epithelium the intermediate layer sheds the cornified cells. Despite these differences, if it could be established that the oral mucosa undergoes rhythmic changes as does the vaginal mucosa, the oral smear might prove to be another means of indicating certain hormonal conditions. In some cases, this method could present advantages over the vaginal smear as it is a simpler operation, involves fewer technical difficulties, could be applied to both men and women, and does not arouse the negative emotional response which often hampers or contraindicates the taking of vaginal smears.

Observations were made on a group of women complaining of orolingual pain. These patients were placed on estrogen therapy and were studied in connection with the orolingual problem, which is reported elsewhere (1). This group, plus others in estrogen deprivation states, also provided the experimental material for the present report, on the theory that the process of epithelial restoration could be more readily followed by means of oral and vaginal smears in a series receiving substitution therapy than in women with normal estrogen function.

We were guided by Papanicolaou's original work on vaginal smears (2) together with later amplifications by Papanicolaou and Shorr (3) (4) and others (5,6, 7, 8, 9). The conclusion to be drawn from these studies is that the proper use of daily vaginal smears enables the investigator to follow

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major deviations in ovarian hormone functions as well as important changes during the menstrual cycle.

In order to emphasize the status of hormonal action on the oral mucosa, a summary of certain experimentation is interjected before describing the present problem further.

SOME PREVIOUS STUDIES OF HORMONAL EFFECTS ON ORAL MUCOSAE

One of us (Ziskin) studied the effects of ovarian and other hormones on the oral mucous membranes.[1] Estrogens were injected into rhesus monkeys

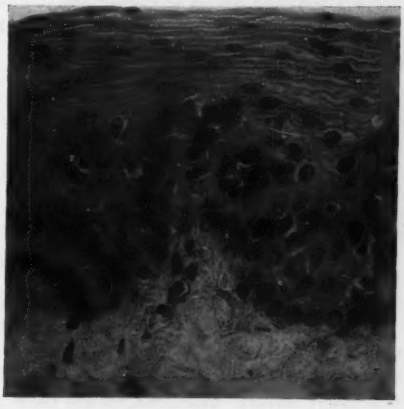

FIG. 1. Monkey 209. Female macacus rhesus. Normal mucous membrane. The keratin layer is absent, as in the human tissue. ×750.

Note: All tissue sections (Figs. 1 to 13, inclusive) are from female macacus rhesus monkeys weighing from 2 to 4 kilograms. The term "gingival," as used here, refers to the hard, pink gums; the term "mucous membrane," to the tissue immediately beyond the hard, pink gums and extending to the muco-buccal reflection; the term "cheek mucous membrane," to the lining of the cheek.

in the following categories: 1) castrated, 2) hypophysectomized, 3) normal immature, 4) sub-adult, 5) adult female, and 6) adult male. In all six groups the clinical result of estrogen injections was the production of gingival hypertrophy. This was seen by an amelioration of gingivitis, if present, and by a thickening and blanching of the gingivae.

The usual gross dermal changes, such as reddening and wrinkling of the sex skin, were observed. Microscopically, the gingival changes seen clinically proved to be hyperplasia (Figs. 16 and 17). Normal oral mucous membranes do not show surface keratin as do the gingivae (Figures 1, 14, 15); yet, after the estrogen injections, there was a definite layer of keratin on all mucous membranes (Fig. 6). The normally keratinized gingivae became hyperkeratinized as evidenced by a thickening and maturation of the sur-

[1] See References (10, 11, 12, 13, 14). This review is included as background because it presents certain factors influencing the epithelial structures of the oral mucosae. Since the major interest here centers around the absence, presence or modification of the keratin layer, the photomicrographs included were selected to illustrate these conditions.

face keratin in some (Fig. 17), and the formation of epithelial pearls in the prickle cell layer (Figs. 5, 16, 17). Mitosis was slightly increased.

The described changes were seen in all categories, proportional to dos-

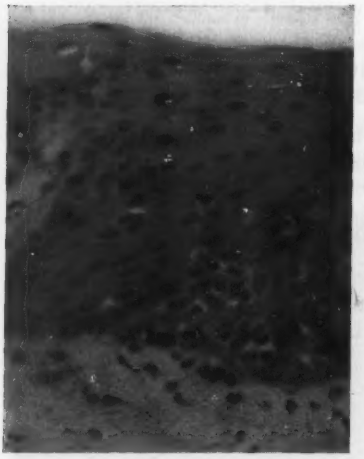

FIG. 2. Monkey 214. 2½ months castrate. Mucous membrane. Surface keratin is absent. The prickle cells are smaller than normal, irregular in size, hyperchromatic, and pyknotic. The nuclear membranes are prominent. Compare with Fig. 1. ×400.

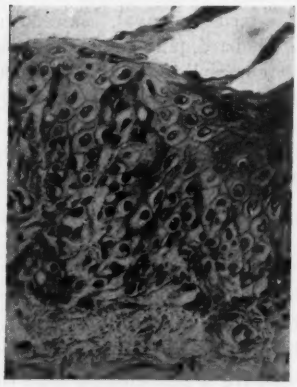

FIG. 3. Monkey 233. 8 months castrate. Mucous membrane. Fragmentation, pyknosis, and lysis of cells; and generally marked tissue alteration of a degenerative nature. The changes are more severe than in Fig. 2. ×540.

FIG. 4. Monkey 238. Hypophysectomized (complete) 47 days. Mucous membrane. Shows pyknosis, lysis of cells, fragmentation of tissue. Similar but more emphatic changes than seen in Fig. 3. ×1340.

age (within the limits of the capacity of the tissues), and were most prominent in those animals under treatment for the longest period of time.

To test further the estrogen effect, pyridine extracts of the anterior pituitary gland, an extract of the urine of castrated women, and an extract from blood serum of pregnant mares (equine gonadotropin), all known to have an ovarian follicle stimulating effect, were injected into

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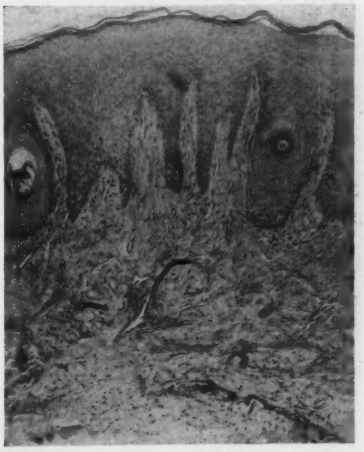

FIG. 5. Monkey 208. Castrate. Shows the gingivae after the injection of 135,000 R.U. estrogen in 79 days. Note the hyperplasia of both the epithelium and connective tissue and the presence of epithelial pearls. ×150.

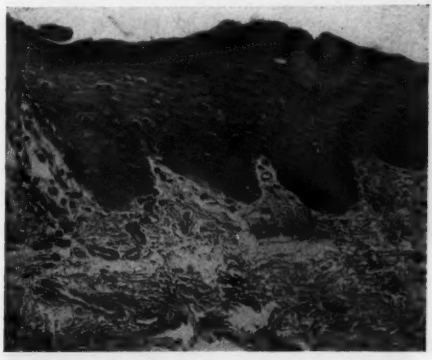

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FIG. 6. Same animal as in Fig. 5. Cheek mucous membrane. Shows hyperplasia and presence of a keratin layer where none is seen ordinarily.

baby monkeys, into young mature monkeys and into hypophysectomized monkeys (completeness of hypophysectomy demonstrated by serial sections of the capsule). In all, the characteristic estrogenic changes described above were induced (Figs. 7 and 8).

The oral mucous membranes of castrated and hypophysectomized monkeys without estrogen replacement were also considered. In the former, the changes varied in degree, depending on the length of the period of castration. The whole tissue stained faintly and became fragile, being torn easily while sectioning. The normal keratin-bearing areas showed parakeratosis, thinning, or loss of surface keratin. The prickle cells lost their horizontal arrangement. The cells were columnar in shape and

disposed in vertical rows. The intercellular spaces were widened, giving them undue prominence. The nuclei were shrunken and pyknotic and a moth-eaten appearance was seen in many areas. The basal cells were small,

FIG. 7. Monkey 210. Shows the gingivae after treatment with gonadotropic hormone extracted from the urine of castrated women. (13,800 cc. urine equivalent injected in 37 days.) The changes are hyperplasia and hyperkeratinization. Note similarity to Figs. 5, 16, and 17. ×150.

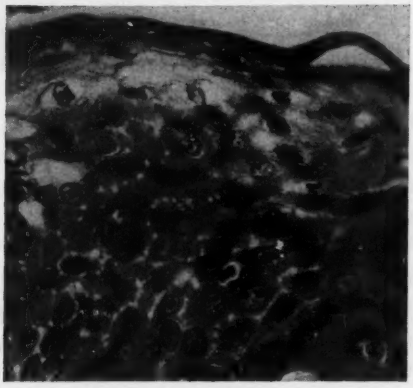


FIG. 8. Monkey 487. Mucous membrane. Hypophysectomized (complete). Treated with 5,675 units of an extract containing the gonadotropic hormone of pregnant mare serum (equine gonadotropin) over a period of 32 days. Note the presence of an abnormal layer of keratin on the surface. ×1020.

irregular in size, with hyperchromatic nuclei. The whole tissue was narrower (Figs. 2 and 3). The changes following hypophysectomy were similar to the foregoing, but more severe (Fig. 4).

Several factors were found to modify the estrogen effect on the oral tissues of monkeys. Prominent among these was chorionic gonadotropin. Microscopically, the keratin layer of the epithelium appeared diminished in thickness or absent entirely. A zone of parakeratosis was sometimes seen in this area. Extensive hydropic change was seen in the prickle cell layer, with pyknotic nuclei toward the surface. Hyperplasia of the germinal layer was observed. The rete cones were elongated, pointed, and split frequently (Figs. 9 and 10). The chorionic gonadotropin may be partly re-

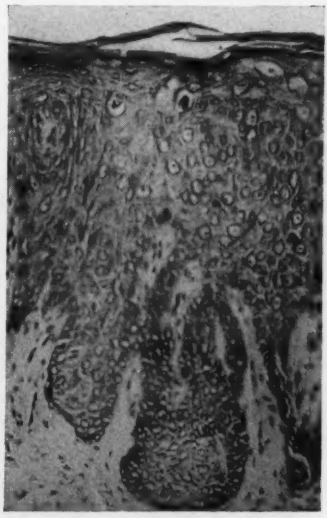


FIG. 9

FIG. 10

FIG. 9. Monkey 123. The pre-experimental gingivae. ×300. See Fig. 10.

FIG. 10. Gingivae of same animal as in Fig. 9 after treatment with a crude extract of pregnancy urine (chorionic gonadotropin) for 68 days. The change consists of loss of surface keratin replaced by a parakeratosis. Marked hyperplasia is also seen. ×160.

sponsible for similar changes in the oral mucosa of pregnant women (15), (Figs. 21 and 22).

The effects of progesterone in both castrated and normal female monkeys were also tested. The epithelial changes consisted chiefly of a diminution or alteration in the keratin layer and an altered appearance of the prickle cells, mainly in the upper half of the layer. In both groups, prickle cell nuclei were pyknotic, shrunken, irregular in size and outline, and, in some instances, fragmented. The changes noted were more extreme in the castrated animals.

These experiments showed that estrogens in large quantities produced hyperkeratinization of the oral mucous membranes while other internal

secretions or their absence resulted in modifications of the keratin formation.

Changes in gingivae and other oral mucous membranes, similar to those seen in the monkeys, were found in the biopsies of a group of women receiving estrogen injections for gynecological disorders (Figs. 16 and 17).

Oral biopsies from clinical cases with other endocrine disturbances substantiated this evidence. For example, in a patient with Addison's disease a hyper-estrogen condition is suggested, the altered estrogen metabolism affecting the surface keratin (Fig. 18). Conversely, in a child of 7 years with

FIG. 11. Monkey 471. Cheek mucous membrane of normal animal injected with 92 mg. of progesterone in 30 days. Shows irregular outline and pyknosis of surface cells, and increased prominence of nuclear membranes. Outline of nuclei in germinal cells is also irregular. ×1000.

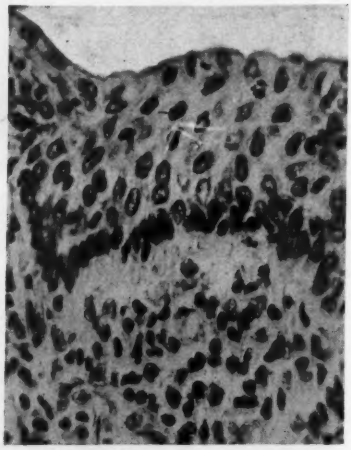


FIG. 12. Same animal as in Fig. 11. Vaginal mucous membrane. Shows irregular outline of nuclei and increased prominence of nuclear membranes. ×1000.

adrenal hyperplasia, pseudohermaphrodism, hirsutism and accelerated osseous development, the gingivae showed a lack of normal surface keratin (Fig. 19). This lack was also noted in a female, age 21, with glandular dystrophy (of unknown etiology), hirsutism and secondary amenorrhea of recent origin (Fig. 20).

The inferences drawn from this research suggested that stained scrapings

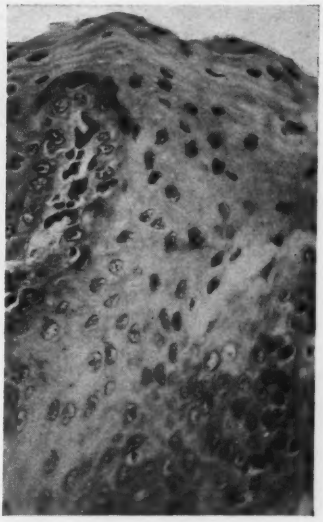


FIG. 13. Monkey 459. Castrate. Injected with 63 mg. of progesterone in 31 days. Gingival section shows irregular outlines of prickle cell nuclei. ×710.

FIG. 14. D. W. Female, age 26. Normal gingivae, 18th day of menstrual cycle. This photomicrograph is included to illustrate the histology of the normal human gingivae. The normal monkey's gingivae are similar except that the layer of granulosa cells is absent. ×117.

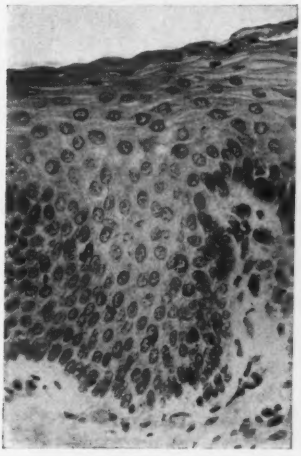


FIG. 15. C.S. Male, age 24. Normal mucous membrane. The keratin layer is absent, the surface being composed of flattened nuclei. ×560.

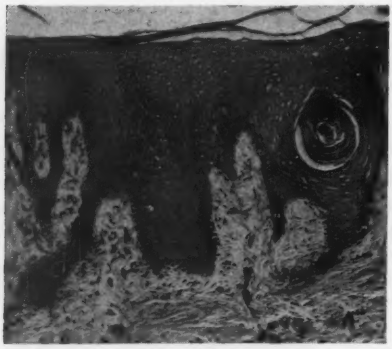


FIG. 16. B.H. Female, age 31. Gingivae, after injections of 85,000 R.U. estrogen in 8 weeks for the treatment of secondary amenorrhea. The tissue shows hyperplasia, hyperkeratinization and epithelial pearls. ×160.

FIG. 17. B.H. Female, age 41. The gingivae after diethylstilbestrol therapy for 2 years for a gynecological disorder. Note the presence of a heavy keratin layer on the surface, hyperplasia, and epithelial pearls. ×175.

FIG. 18. J.R. Male, age 29. The gingivae from a case of Addison's disease. Hyperplasia and hyperkeratinization are in evidence. The implication is that because of adrenal cortical insufficiency, the modification of the estrogenic effect is absent, thus making more endogenous estrogen available to the gingivae. ×160.

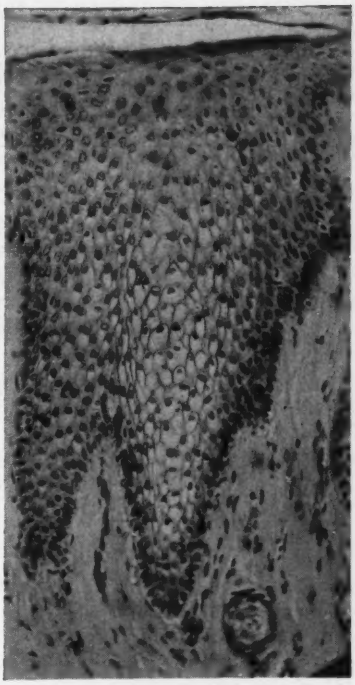


FIG. 19. C.A. Male, age 7. A case of adrenal hyperplasia and pseudohermaphroditism; bone age 25 years; infantile ovaries and uterus removed; no testicular tissue found; hirsutism and enlarged clitoris gave masculine appearance. The gingivae show absence of surface keratin. The round pyknotic nuclei seen on the surface together with the deeper-staining cytoplasm may constitute parakeratosis. Pyknotic nuclei and hydropic change appear in the prickle cell layer. The basal cells are small, irregular and widely spaced. The implication is that the excessive cortical secretion has a modifying effect on the endogenous estrogen so that the estrogenic effect on the gingivae is reduced or absent. ×336.

←

FIG. 20. A.T. Female, age 21. A case of hirsutism with endocrine gland dysfunction of unknown origin, and secondary amenorrhea of recent origin. The gingivae show an absence of surface keratin which may be attributed to a modification of the available endogenous estrogen. ×224.

from gingival surfaces might be useful in evaluating endogenous estrogen levels, and this hypothesis constitutes the basis for the present comparative study.

METHODS

The oral epithelial smears consisted of scrapings taken from the surface of the cheek mucosae and from the keratinized gingivae by lightly passing a thin flat metal blade (spatula) over the tissues.[2] The vaginal smears

[2] There are various levels of keratinization in the mouth: the hard palate would rate 4 plus; the hard pink gums, 3 plus; and the cheek mucosa (which normally does not have

were made by inserting into the vagina a glass tube to which was attached a rubber bulb, as described by Papanicolaou (2). The cells shed from the vaginal walls were thus drawn into the tube.

The cells from both areas were spread on glass slides and fixed in alcohol-ether (50–50). First, the oral smears were stained by Weinman's method

TABLE 1. STAGES OF CORNIFICATION AND CELL TYPES IN ORAL AND VAGINAL* EPITHELIAL SMEARS

Stage of Cornification	Percentage of Cornification	Predominant Types of Cells and General Appearance of Smear
Stage 0 (Atrophic smear)	None	Pale blue, non-cornified cells with large vesicular nuclei predominant. Many small basal cells with blue cytoplasm in vaginal smear.
Stage I (Resembles menstrual or early postmenstrual smear)	1–15%	Many non-cornified cells with some cornified forms with purplish cytoplasm and some smaller nuclei. Cells generally clumped. Many W.B.C. and cellular debris in vaginal smear.
Stage II (Resembles follicular or copulative smear)	15–40%	Many cornified red cells with small dark pyknotic nuclei. Remaining cells purplish, precornified forms.
Stage III (Maximum estrogen response. Resembles ovulatory smear)	40–95%	Most cells cornified. Cells distinct and separate. Vaginal smear "clean" with minimum number of W.B.C.
Stage IV (Resembles estrogen decline in the progestational phase)	80–40%	Both cornified and noncornified cells. Fragmented, anucleated, degenerative forms. In vaginal smear, cellular debris and W.B.C. return.

* Compare description of cells with that of DeAllende, I.L.C., Shorr, E., and Hartman, C. G., "A Comparative Study of the Vaginal Smear Cycle of the Rhesus Monkey and the Human," Carnegie Institution of Washington. Publication 557. Contributions to Embryology. XXXI, 1–26, December 22, 1943.

(16), using the the Ernst modification (17) of the Gram stain; and the vaginal smears were given the Shorr modification of Masson's trichrome stain (18). Later, the Gram stain was discarded and the Shorr method was used for both.

The oral and vaginal smears were taken simultaneously from normal

a definite keratin layer) occasionally 0 to 1 plus. In this series, scrapings from the cheek were used wherever possible because they took the Shorr stain as effectively as the vaginal cells and, therefore, facilitated comparison. Gingival smears were taken when indicated.

menstruating women. Readings were made by each of us, independently, and then compared and uniformly recorded. After ascertaining that the major changes in cornification of the vaginal smear were reflected in the oral smear, a series of cases in various endocrine states was studied in greater detail.

In cases where the menstrual cycle was absent, changes in cornification resulting from estrogen or other therapy were recorded in stages graded I,

TABLE 2. EFFECT OF ESTROGENIC TREATMENT ON EPITHELIAL SMEARS OF TEN POSTMENOPAUSAL WOMEN*

Case	Age Years	Menopause Years	Stilbestrol mg.	Benzestrol mg.	Estradiol Benzoate rat units	Stages of Cornification					
						Before Treatment		During Treatment		After Treatment	
						Vag.	Oral	Vag.	Oral	Vag.	Oral
No. 7, A.G.	54	5.5	21				I		II	I	I
No. 8, C.H.	56	15	50				0	III	II		
No. 9, A.A.	55	10	46				I	II	II		I
No. 10, R.S.	56	9	12				I	III	II		I
No. 11, S.Y.	52	3	9		40,000	II	III	III	II		
No. 12, B.H.	56	10	16	56		I	0	III	I		0
No. 13, E.C.	55	12	13	75			I	III	I		I
No. 14, F.S.	58	8		200	100,000		I	III	1		I
No. 15, B.P.	58	6		80	40,000	I	I		II		I
No. 16, J.E.	54	½		80	90,000	I	I	II	II	I	I

* Stilbestrol was given in doses of 1 mg. daily; benzestrol 2 mg. daily; and estradiol benzoate 10,000 rat units weekly. Duration of treatment may accordingly be estimated from the total doses listed.

II, III and IV, since the phases of the menstrual cycle did not exist. The cornification stages are explained in Table 1 and illustrated in Figure 23.

EXPERIMENTAL PROCEDURE

Oral and vaginal smears were compared in a group of 23 women. *Case 1* was that of a girl with secondary amenorrhea; *Case 2* was a normal pregnancy; *Cases 3 to 6*, inclusive, were in menstruating women with fairly normal cycles; *Cases 7 to 16*, inclusive, comprised ten middle-aged women, all menopausal, presenting the complaint of oral burning (tongue or gums) or dryness of the mouth and throat; and seven women of menopausal age (*Cases 17 to 23*, inclusive) without oral complaints, to serve as controls for the ten cases of glossodynia.

The vaginal and oral smears, treated with the Shorr stain, presented certain differences as well as some features in common. The dissimilarities were as follows:

1) No basal cells were seen in the oral smears.

2) The cytoplasm in the precornified oral cells showed purple granules and stained more deeply than the vaginal. The oral cells and nuclei were

FIG. 21. B.E. Female, age 27. Eighth month of pregnancy (multipara). A section from the gingivae. Note loss of surface keratin, hyperplasia, downgrowth of rete cones, and inflammatory exudate in lamina propia. ×150.

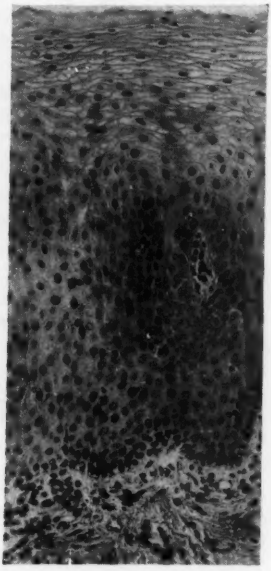


FIG. 22. Same case as in Fig. 21. A section from the mucous membrane. Changes are similar to those in Fig. 21. ×336.

smaller than those in the vaginal smears and the nuclei were more pyknotic. (The oral precornified cells are the counterpart of those vaginal cells characteristic of the copulative phase of the menstrual cycle.)

3) The white blood cells seen mostly at ovulation in the vaginal smear were rarely found in the oral smear.

The following are the main points of similarity:

1) The non-cornified cells of the oral smears showed a pale grey-blue cytoplasm with large vesicular nuclei resembling the vaginal cells found most plentifully in the menstrual and postmenstrual smears.

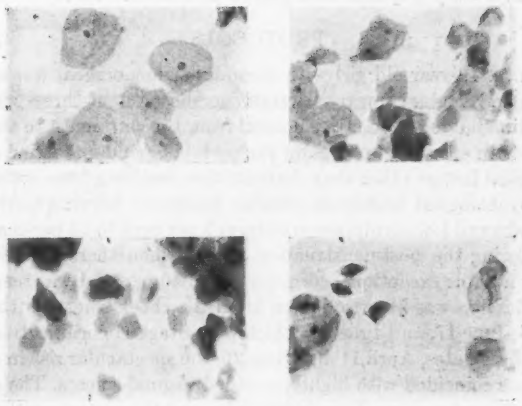


FIG. 23. Oral epithelial smears stained with the Shorr trichrome stain and illustrative of the four stages in cornification. Stage I: menstrual and postmenstrual phase. Stage II: copulative phase. Stage III: preovulatory and ovulatory phase. Stage IV: estrogen withdrawal phase; and degenerative forms.

2) The fully cornified oral cells were for the most part seen to be discrete and red (eosinophilic). Some had dark, pyknotic nuclei, others were anucleated, and still others showed a vacuole in the center where the nucleus had dropped out, or a clear halo around a degenerated nucleus. (These oral cells are the counterpart of the vaginal cells characteristic of the time of ovulation. They are found most commonly in scrapings from the gums.)

3) The postovulatory or progestational phase of the cycle was typified in both groups by degenerated, folded, and fragmented cells, usually clumped together (Fig. 23). (The histological sections from progesterone treated monkeys (Figs. 11, 12, 13) also show distorted and fragmented cell outlines and may explain the forms described here.)

PROTOCOLS

Case 1. G. A., a 19-year-old girl with secondary amenorrhea, was studied for seven months by oral and vaginal smears. Her amenorrhea was of three years duration, the menstrual function having been fairly normal from the thirteenth to the sixteenth year. Medical study, with special emphasis on the metabolic and endocrine aspects, failed to reveal any physical factors other than malnutrition resulting from anorexia. There were evidences of psychological factors as possible causes of the amenorrhea. Most of the vaginal smears showed low cornification of from 5 per cent to 15 per cent, and resembled smears taken during the postmenstruation period when there is little estrogen effect. There were two notable exceptions: cornification of 40 per cent was seen on April 11 and June 26. Cornification was likewise low in all of the cheek smears with four exceptions: April 11, May 3, June 17, and June 26, which were Stage II cornification. It is significant that on two of these dates, April 11 and June 26, the spectacular rise in cornification seen in the oral smears coincided with highly cornified vaginal smears. The gums throughout were swollen, soft, and spongy. They bled easily, and the surface epithelium was readily shed. This was accounted for by low cornification and, probably, depressed estrogen effect. Scrapings for the epithelial smears were taken with ease in contrast to the usual difficulty encountered in cases of hard, firm gums. Although the cause of the cornification peaks is obscure, it is pertinent that both mucosae were affected simultaneously in these two instances.

Case 2. R.F., a woman 29 years of age. An uncomplicated pregnancy was studied by means of vaginal and oral smears taken at weekly intervals from the first month until five weeks after delivery, except that during the crucial two weeks starting the day before delivery they were a daily procedure. The vaginal smears seen during the 10-day period following delivery were obscured by blood. All others were satisfactory.

In the first month of pregnancy, the vaginal smears showed a cornification of from 15 per cent to 20 per cent (Stages I and II), and were "dirty" with many white blood cells and debris. As pregnancy progressed, cornification fell, until by the sixth month cornified cells were rare; but they increased to about 1 per cent during the two weeks before delivery. The vaginal smears became cleaner, especially during the seventh and eighth months, but debris and white blood cells increased again in the last month. During the first eight months the cells became progressively smaller and more folded and wrinkled, with an increasing predominance of the "oyster" and "navicular" cells typical of pregnancy. Five days before delivery the cells appeared plumper; the day before delivery, at the onset of labor, they were vesicular. Little could be ascertained from the vaginal

smears for several days after delivery, except that cornification was low. On the ninth day post partum, cornification suddenly increased to about 10 per cent and remained at approximately that level for the balance of the experimental period. Basal cells were seen during the third post partum week.

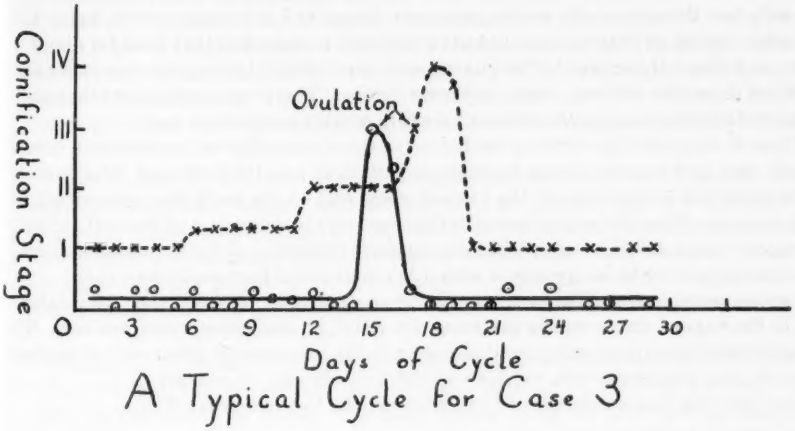
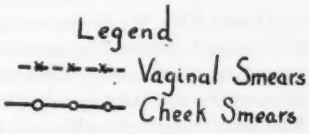


FIG. 24



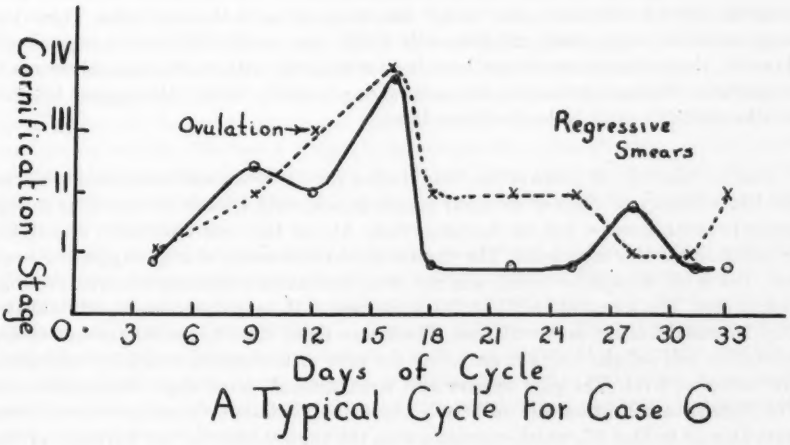


FIG. 25

The cheek smears showed low cornification (about Stage O to I), as in normal women, for six months, after which there was a drop (Stage O). The latter level remained more or less constant during the rest of the observation time. The cheek mucosa, being the least keratinized of the oral mucosae, did not seem to reflect the estrogen changes in this case. However, interesting alterations were seen in the gums. Here cornification was unusually low throughout the entire pregnancy (Stage O-I as compared with Stage III normally). It rose to Stage II ten days after delivery, remained at that level for about 3 weeks, and then fell to Stage I. The gum smears were "dirty" throughout except for the first three days after delivery, when they were "clean." There was an increased tendency to gingival bleeding which persisted until the fifth or sixth postpartum day.

These findings disclose striking similarities in the abnormally low cornification noted in both gum and vaginal smears throughout gestation, and the increased cornification on the ninth day postpartum in the vaginal smear and on the tenth day postpartum in the gum smear. Since the vaginal cornification rose prior to delivery, and the oral did not, the vaginal smear would seem to be a more sensitive indication of the hormonal changes, and would appear to be in agreement with other methods of hormonal assay (19).

Another outstanding difference was the presence of the "oyster" and "navicular" cells in the vaginal smear during gestation, for which no oral counterpart was seen. To our knowledge there is no adequate description in the literature of either oral or vaginal smears during pregnancy with which these observations may be compared.

The following four cases represent observations on the menstrual cycle.

Case 3. Mrs. R., 26 years of age, was studied for three months by means of daily oral and vaginal smears. The patient had a menstrual history of average regularity. Physical examination revealed no abnormalities. One cycle is reported here because the vaginal smears followed the typical pattern described for the normal menstrual cycle (Fig. 24). The peak in estrogen effect on the cheek smears was seen one day before presumptive evidence of ovulation was manifest in the vaginal smear. During the last few days of the cycle the cheek smears were not "clean" and clear-cut as in the beginning. There were many wrinkled, fragmented, anuclear cells which were comparable to the vaginal cells. However, these changes would not have been convincing without the vaginal smears for comparison. The gum smears in this case were unusually "dirty" throughout the three months and little could be learned from them.

Case 4. Miss M., 30 years of age, was studied for six weeks with semi-weekly smears. She had a history of years of irregular menstruation, with periods of from four to eight weeks between menses, but no dysmenorrhea. At one time she had taken an estrogen preparation for this complaint. The vaginal smears showed a change suggesting ovulation, but a full copulative smear was not seen, indicating a possible abnormal estrogen metabolism. The smear of Day 12 of the cycle was of the copulative stage, while that on Day 15 was definitely postovulative. There was more than the usual amount of fragmentation and cellular breakdown before the next menstruation, probably indicating a low hormone level. The gum smears also went through some slight discernible cycle. The highest cornification was on Day 8, preceding ovulation. Cornification was lowest from Day 18 to Day 27, which correlates with the vaginal phase of low hormone production and degeneration.

Case 5. Mrs. H., a 37-year old woman, had oral and vaginal smears taken daily for a month. She had a normal mental, physical, and menstrual history, except that recently she had been experiencing some pain in her breasts in mid-cycle. It was estimated from the vaginal smears that ovulation occurred about Day 12 in one cycle, but the copulative phase was not well developed. There were regressive changes on Days 7 and 8, and also during the last third of the cycle. The change from the copulative to the progestational phase in the gum smear was judged to occur on about Day 10. In the cheek smear there was a drop in cornification after Day 8 as well as an increase in bacteria, debris, and cell fragmentation. The highest cornification was evidenced on Day 9 in the gum smear and three days later in the vaginal smear, thus suggesting a general correlation.

Case 6. Mrs. A., 31 years of age, was studied psychiatrically for 18 months. Oral and vaginal smears were taken simultaneously for two months (Fig. 25). Changes in the vaginal epithelium were comparatively easy to follow and the ovulatory peak was fairly well defined. The cheek smears showed a peak of cornification and increased "cleanliness" during a four-day period which corresponded in time to ovulation, according to the vaginal smears. The progestational phase began simultaneously in both series of smears.

Cases 7 to 16, inclusive, comprise ten middle-aged women whose chief complaint wa either "burning" of the tongue (glossodynia), "burning" of the oral mucosae, or "dryness" of the mouth or throat. The usual causes listed for these complaints, such as pernicious anemia, achlorhydria, etc., were ruled out by physical examination.

The patients were accepted in the order of their appearance at the clinic, no attempt being made at selection. All were past the menopause and in the fifth decade of life. They were given an estrogenic substance: diethylstilbestrol, benzestrol,[3] or estradiol benzoate. In those instances where more than one drug was administered, the medications were given at successive stages of the treatment, not simultaneously. Generally, the dosages were as follows: Diethylstilbestrol, 1 mg. per day, orally; benzestrol, 2 mg. per day, orally; estradiol benzoate, 10,000 R.U. a week, parenterally (Table 2). Oral and vaginal epithelial smears were taken at weekly intervals, or more frequently, and therapy was extended over a period of from two to three months or longer.

The vaginal smears in this experimental series were of the atrophic variety. The gingival smears showed a reduction in keratinization when compared with those of normal young women. Whereas a Stage III keratinization is characteristic of firm, healthy gingivae, both the experimental cases (7 to 16) and control groups (cases 17 to 23) showed low gingival keratinization (Stages I to II). The gross appearance of the gingivae varied little in the two groups.

In all the treated cases (7 to 16) there was evidence of adequate vaginal response to the estrogen, with marked increase in cornification. Seven of the gingival smears in this group revealed an increase in cornification; three showed none. The cheek smears gave evidence of increased cornification in 7 cases, but the rise did not generally coincide with the vaginal response (it came from two to four weeks later) and was less intense. In both sets of smears, those patients receiving the diethylstilbestrol gave the best results whereas the change in the cheek smears was minimal or absent in those treated with benzestrol or estradiol benzoate.

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[3] Product of Research Laboratories of Schieffelin & Company.

CONCLUSIONS

From these studies it may be concluded that:

1) The oral and vaginal epitheliums show parallel changes in degree of cornification during the menstrual cycle, and during estrogenic treatment in deprivation states.

2) Slight hormonal changes are more readily discernible in the vaginal smear than in the oral smear. Consequently, cyclic hormonal changes as seen in the normal menstrual cycle may be better evaluated by means of the vaginal smear.

3) Estrogen deprivation states produce low cornification in both sets of smears, while the response to estrogenic treatment is more strikingly demonstrated in the vaginal than in the oral smear.

4) The vaginal smear, while not an infallible criterion, is a better indicator of ovulation time than the oral smear.

5) The oral smear, despite its technical advantages, cannot replace the vaginal smear as a method of determining hormone levels in women.

ACKNOWLEDGMENT

We wish to express our thanks to Doctors George E. Daniels and S. N. Rosenstein for their assistance in furthering this study. We are also indebted to the Human Sex Biology Gift of Columbia University for contributions toward the compilation of these data.

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CHEMICAL ASSAY FOR "CORTIN"

DETERMINATION OF FORMALDEHYDE LIBERATED ON OXIDATION WITH PERIODIC ACID

WILLIAM H. DAUGHADAY, M.D.,* HERBERT JAFFE, Ph.D. AND ROBERT H. WILLIAMS, M.D.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts

SEVERAL properties of adrenal cortical hormones have been utilized for their assay. The more common bio-assays have depended upon the ability of urinary extracts in adrenalectomized rats or mice a) to maintain life (1), b) to protect against the effects of cold (2), and c) to promote the deposition of glycogen in the livers of fasting animals (3). Because these methods are tedious and prone to considerable biological variation, efforts have been made to develop chemical assays.

The reducing power of corticosteroids has been made the basis for two chemical assays. One procedure employs relatively crude (4), and the other more purified ketonic urinary extracts (5).

The use of the periodic acid oxidation reaction for the estimation of certain urinary steroids was proposed by Fieser, Fields, and Lieberman (6). Under certain conditions the steroid remnant after oxidation is a 17-ketosteroid which can be measured by the Zimmermann reaction. Talbot et al. have used this reaction for the measurement of certain non-ketonic urinary steroids (7). However, the measurement of newly formed 17-ketosteroids has not proved a reliable method for the estimation of urinary corticosteroids (8).

Lowenstein and his coworkers (9) have proposed a method based on the quantitative measurement of the formaldehyde liberated by the oxidation of urinary extracts with periodic acid. In attempting to repeat the detailed procedure supplied to us by the authors, we encountered considerable difficulty. Reasonably reliable results were obtained with crystalline steroids. However, substances interfering with the color reaction were present in small amounts in commercial adrenal cortical extract and in large amounts in many urinary extracts. Because of this difficulty we have introduced a distillation step whereby the formaldehyde liberated by the oxidation is distilled into a sulfite solution leaving behind most of the interfering substances. When the reaction with chromotropic acid is applied to the dis-

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* Present address: Barnes Hospital, St. Louis, Missouri.

tillate, the color formed is comparable to that obtained with known formaldehyde solutions. The use of this reagent for the determination of formaldehyde in biological fluids was described by MacFadyen (10).

REAGENTS

1. Chloroform, CP.
2. Benzene, CP.
3. Sodium sulfate, anhydrous, CP.
4. Periodic acid reagent: 0.01 M. potassium periodate,[1] in 0.15 M. sulfuric acid.
5. Stannous chloride reagent: Dissolve 6 Gm. of stannous chloride in 10 cc. of hot concentrated hydrochloric acid, dilute with water to a volume of 100 cc. Add a small amount of tin shot to stabilize the solution. Discard when turbid.
6. Chromotropic acid stock solution: 5 per cent chromotropic acid.[2]
7. Chromotropic acid reagent: 4 cc. of chromotropic acid stock solution diluted to 100 cc. with 15 M. sulfuric acid. Make up fresh for every run.
8. Alcoholic sulfite solution: 4 Gm. of sodium sulfite, CP, dissolved in 100 cc. of 1 per cent ethyl alcohol.

METHOD

1. **Extraction:** A 24-hour urine sample is collected with 10 cc. of chloroform as a preservative. The pH of a 200 cc. aliquot is adjusted to about 1.7 with 5 M. sulfuric acid using an indicator paper.[3] Extraction with 200 cc. of chloroform is carried out immediately by shaking in a separatory funnel for five minutes. The emulsion formed is broken by centrifugation and the chloroform layer is drawn off with suction. The remaining urine is re-extracted with 150 cc. and 50 cc. of chloroform successively in a similar fashion. The chloroform extracts are combined and dried over 40 Gm. of anhydrous sodium sulfate for 20 minutes. The solution is then filtered through fluted paper and the sodium sulfate is washed three times with 15 cc. of chloroform.

The extract is next evaporated to a residue (Fig. 1, Residue A) under reduced pressure and a temperature of about 40°C. Residue A is taken up in 10 cc. of benzene and filtered into a 50 cc. florence flask. The original flask is washed twice with small amounts of benzene which are also filtered.

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[1] Obtained from G. Frederick Smith Chemical Co., Columbus, Ohio.

[2] Obtained from Eastman Kodak Co., Rochester, New York. (1,8-dihyroxynaphthalene, 3,6 disulfonic acid, practical.)

[3] "Accutint" paper #20, Anachemia Ltd., Montreal, Canada.

The benzene extract is evaporated to dryness under reduced pressure at about 40°C. to form a residue (Fig. 1, Residue B). It is now possible to take up Residue B in 1 cc. of benzene and transfer it quantitatively to a small centrifuge separatory funnel[4] using two washings of 0.5 cc. of benzene. The final benzene extract with a volume of 2 cc. is partitioned with 5 cc. of distilled water four times, centrifuging at a slow speed each time to

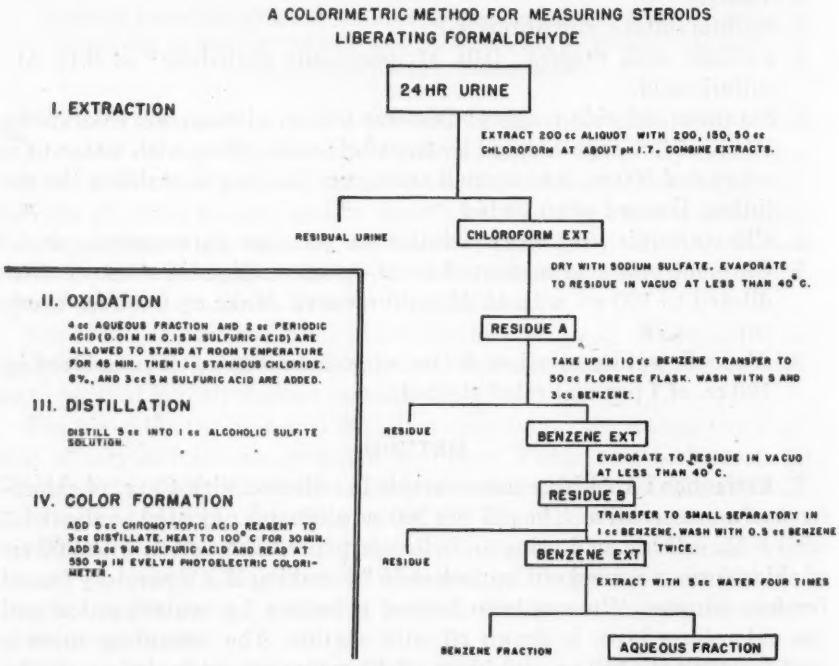
A COLORIMETRIC METHOD FOR MEASURING STEROIDS
LIBERATING FORMALDEHYDE
I. EXTRACTION
24 HR URINE
EXTRACT 200 cc ALIQUOT WITH 200, 150, 50 cc CHLOROFORM AT ABOUT pH 1.7. COMBINE EXTRACTS.
RESIDUAL URINE
CHLOROFORM EXT
DRY WITH SODIUM SULFATE. EVAPORATE TO RESIDUE IN VACUO AT LESS THAN 40°C.
II. OXIDATION
4 cc AQUEOUS FRACTION AND 2 cc PERIODIC ACID (0.01 M IN 0.15 M SULFURIC ACID) ARE ALLOWED TO STAND AT ROOM TEMPERATURE FOR 45 MIN. THEN 1 cc STANNOUS CHLORIDE, 6%, AND 3 cc 5 M SULFURIC ACID ARE ADDED.
III. DISTILLATION
DISTILL 5 cc INTO 1 cc ALCOHOLIC SULFITE SOLUTION.
IV. COLOR FORMATION
ADD 5 cc CHROMOTROPIC ACID REAGENT TO 3 cc DISTILLATE. HEAT TO 100°C FOR 30 MIN. ADD 2 cc 9 M SULFURIC ACID AND READ AT 550 mμ IN EVELYN PHOTOELECTRIC COLORIMETER.
RESIDUE A
TAKE UP IN 10 cc BENZENE TRANSFER TO 50 cc FLORENCE FLASK. WASH WITH 5 AND 3 cc BENZENE.
RESIDUE
BENZENE EXT
EVAPORATE TO RESIDUE IN VACUO AT LESS THAN 40°C.
RESIDUE B
TRANSFER TO SMALL SEPARATORY IN 1 cc BENZENE. WASH WITH 0.5 cc BENZENE TWICE.
RESIDUE
BENZENE EXT
EXTRACT WITH 5 cc WATER FOUR TIMES
BENZENE FRACTION
AQUEOUS FRACTION

FIG. 1. The extraction and assay procedure in outline form.

break any emulsion. The combined aqueous fraction has a volume of 20 cc.

2. **Oxidation:** Two cc. of periodic acid solution are added to 4 cc. of aqueous fraction and also to 4 cc. of distilled water which is carried through the assay as a blank. Oxidation is allowed to proceed at room temperature for 45 minutes. Excess oxidant is removed by the addition of 1 cc. of stannous chloride solution, forming a white precipitate.

3. **Distillation:** The oxidation mixture is transferred quantitatively to a small distilling flask[5] with three 1 cc. washings of 5 M. sulfuric acid, bring-

[4] A heavy glass separatory funnel of 15 cc. capacity designed to fit a centrifuge cup made by Macalaster Bicknell Co., Cambridge, Mass.

[5] We have used a small all glass distillation apparatus which may be obtained from Macalaster Bicknell Co., Cambridge, Mass.

ing the total volume in the flask to 10 cc. Distillation is carried out by heating with a micro burner until slightly less than 50 per cent of the solution has been carried over. The distillate is collected in a 15 cc. graduated centrifuge tube to which is added 1 cc. of sulfite solution. The tip of the condenser is placed beneath the surface of the solution to prevent loss. The total volume in the receiving tube is adjusted to 6 cc. by the addition of distilled water.

4. **Color formation:** Five cc. of chromotropic acid reagent are added to 3 cc. of distillate in an Evelyn colorimeter tube. The tubes are heated to 100° C. in a water bath for 30 minutes and then cooled to room temperature. The volume of solution is brought up to 10 cc. by the addition of 2 cc. of 9 M. sulfuric acid and the optical density is measured in an Evelyn photoelectric colorimeter. The galvanometer is adjusted to 100 per cent transmission with the 550 mu filter, using the blank carried through the oxidative procedure mentioned above.

5. **Calculations:** On a weight basis the amount of formaldehyde formed by the oxidation with periodic acid should be approximately between 8 and 10 per cent of the amount of steroid present, assuming that one mole of steroid liberates one mole of formaldehyde. The conversion factor which we have used in our calculations represents a 7.6 per cent liberation of formaldehyde. In view of the indeterminate nature of urinary corticosteroids it is felt that this approximation is sufficiently accurate for the present. After the isolation and identification of the compounds from the urine it may be possible to select a more accurate standard. The term "cortin" has been retained because of historical precedent, although "formaldehyde liberating compounds" would be more accurate.

Thus:

$$\text{Cortin, mg./day} = \frac{(D)\ (K)\ (10)\ (V)}{200}$$

(D) = optical density = $2 - \log G$
(K) = conversion factor of optical density to milligrams of cortin = 0.158
(10) = dilution factor of method
(V) = total volume of 24 hour urine
(200) = aliquot of urine extracted

RESULTS

The extraction procedure we have used conforms in principle to that of Venning, Kazmin and Bell (11) as modified by Lowenstein (12). The extraction is not exhaustive and no doubt some losses occur, but a uniform technique has been utilized throughout so that the relative differences ob-

served probably are significant. It has been demonstrated that acidification results in almost a twofold increase in the amount of biologically active corticoids (8, 11). We have extracted aliquots of the same urine sample at three different pH values and the results are presented in Table 1. It is evident that considerably increased amounts of formaldehyde liberating compounds may be extracted at lower pH values. Heard, Sobel and Venning (4) have demonstrated a similar increase in urinary steroids when measured by their reducing power. The differences in the results obtained by us and Talbot et al. (5) may be explained by the fact that the latter investigators used unacidified urine and used Girard's ketone reagent for purification of the extract. The reason why pH affects the yield of urinary cortin remains obscure. It has been suggested that a labile conjugate of the corticosteroids may be susceptible to such mild hydrolysis (13). The use of strenuous hydrolysis, i.e. concentrated hydrochloric acid and boiling for 7 minutes as carried out for the extraction of 17-ketosteroids, destroys most of the formaldehyde forming substances. This agrees with the observations made using different methods of assay (2, 4).

TABLE 1. THE EFFECT OF pH OF EXTRACTION ON THE RECOVERY OF FORMALDEHYDE LIBERATING COMPOUNDS

Aliquot #	pH	Cortin, mg./day
1	1.7	1.2
2	1.7	1.0
3	3.5	0.75
4	5.4	0.65

The extract has not been washed with water and dilute alkali to remove estrogenic substances and other phenols and pigments, since estrogens do not liberate formaldehyde with periodic acid and urinary pigments do not enter the distillate and therefore do not complicate the procedure.

It is believed that the partition between water and benzene increases the specificity of the method since the property of successive solubility in chloroform, benzene and water is not shared by many compounds capable of liberating formaldehyde. The volume relations of our partition resemble those of Talbot, Saltzman, Wixom and Wolfe (5). These authors have investigated the behavior of five crystalline corticosteroids in such a partition. 17-Hydroxycorticosterone and 17-hydroxydehydrocorticosterone passed quantitatively into the water. Corticosterone and dehydrocorticosterone entered the water only to the extent of 38 and 24 per cent respectively. Desoxycorticosterone remained almost exclusively in the benzene. From these data it appears that water extracts the more oxygenated active corticosteroids from the benzene while compounds with four

oxygen atoms are incompletely removed and those with three oxygen atoms remain almost exclusively in the benzene. Solubility in water is also determined to a large extent by substituents at the 3 position and saturation or unsaturation at the 4–5 position. The partition is not specific for 11-oxy compounds because Δ^4-pregnene-17:20:21-triol-3-one partially enters the aqueous phase (4). We have confirmed the above observations in respect to 11-desoxycorticosterone and the aforementioned pregnene-triol.

Because the partition step represents a potential loss of material we have been interested in the amount of formaldehyde liberating compounds which remain in the benzene fraction. Both the benzene and aqueous fractions of five urinary extracts were assayed and the results are presented in Table 2. In determining the formaldehyde liberating compounds in the

TABLE 2. RECOVERY OF FORMALDEHYDE LIBERATING COMPOUNDS FROM AQUEOUS AND BENZENE FRACTIONS OF URINARY EXTRACTS

Urine Specimen #	Aqueous Fraction	Benzene Fraction	Per Cent Extracted
	mg./day	mg./day	
82	0.9	Neg.	100
98	0.9	0.08	92
97	1.3	0.26	83
99	1.6	0.19	90
84	1.8	0.27	87

benzene fraction we have evaporated off the benzene and dissolved the residue in 1 cc. of ethyl alcohol and then diluted to 6 or 10 cc. with distilled water. The alcohol gives a slight color with the assay and it is necessary to allow for this error. From the results it is obvious that most of the formaldehyde forming compounds in these urinary extracts entered the aqueous fraction.

Unfortunately, compounds have not been available for a thorough study of the oxidation reaction with periodic acid. Fieser, Fields, and Lieberman (6) have stated that compounds with a ketol side chain will yield one mole of formaldehyde for each mole of steroid. With a dihydroxyacetone type of side chain the reaction under certain conditions proceeds to form between one and two moles of formaldehyde. With the glycerol type of side chain there is a greater likelihood of the removal of two carbon atoms. With the method we used desoxycorticosterone was found to liberate one mole of formaldehyde. This variability of formaldehyde formation introduces doubt as to the absolute, but not relative, accuracy of the method. It is doubtful if chemical methods measuring the reducing power of these compounds possess any intrinsically greater accuracy because considerable variation exists in reducing power (13).

TABLE 3. EFFECT OF TIME OF OXIDATION ON RECOVERY OF FORMALDEHYDE FROM ALIQUOTS OF AQUEOUS FRACTION

Amount Oxidized (cc.)	Time (min.)	Formaldehyde (micrograms)
4	15	2.4
4	30	2.56
4	45	2.72
4	60	2.72
4	165	3.04

The time of oxidation has been arbitrarily established at 45 minutes. In Table 3 the representative results obtained by oxidation for varying periods of time is given. The reaction proceeds rapidly for about 30 minutes, followed by a gradual progression.

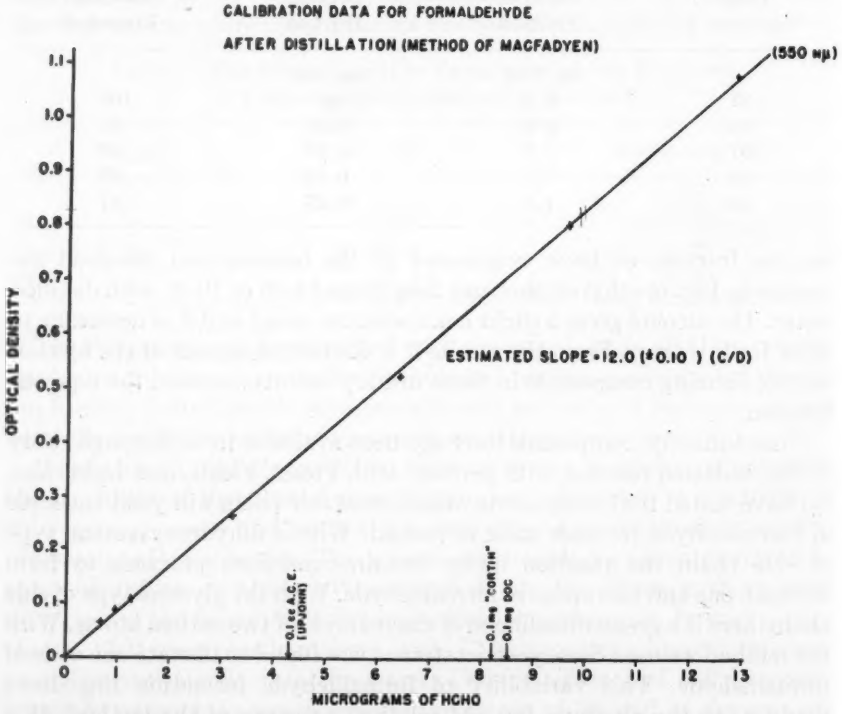

FIG. 2. Hexamethylenetetramine has been used as the source of formaldehyde. The amount of formaldehyde recovered in 3 cc. of distillate is plotted against optical density. The formaldehyde equivalent of 0.1 mg. of 11-desoxycorticosterone, and 0.1 cc. of adrenal cortical extract are compared to 0.1 mg. of our cortin unit.

TABLE 4. EXCRETION OF CORTICOSTEROIDS BY NORMAL SUBJECTS AS MEASURED BY DIFFERENT METHODS OF ASSAY

	Range mg./day	Reference standard
I. Bio-assay		
1. Cold test		
Shipley, Dorfman, Buchwald and Ross (15)	0.5–1.8	11-dehydrocorticosterone
2. Glycogen deposition		
Venning et al. (3)	0.029–0.055 males	
	0.045–0.060 females	11-dehydro-17-hydroxy-corticosterone
II. Chemical Assay		
1. Reducing power		
a. Talbot et al. (5)	0.12–0.34	Corticosterone
b. Salter et al. (16) (modification of II a)	0.14–0.67 males	?
c. Heard, Venning and Sobel (4)	1.1–2.1 males	
	1.0–2.0 females	11-desoxycorticosterone
2. Formaldehyde liberation		
Authors' method	1.0–1.6	Cortin unit, see text

The distillation step separates substances present in urinary extract following periodic oxidation, which modifiy the color reaction. Figure 2 presents the calibration data for our apparatus employing the method of MacFadyen using hexamethylenetetramine as the source of formaldehyde. A linear relation exists over the range of concentration encountered in this procedure, although all of the formaldehyde in the distillate is not recovered.

Results of 120 determinations made on 70 patients and normal subjects are to be presented in a separate communication. The limits of normal have not been established with any finality but it is evident that under the usual conditions of diet and activity the excretion of cortin is between 1.0 and 1.6 mg. a day. The excretion of this material is correlated with known disorders of adrenal function. The normal values obtained by other chemical methods are presented in Table 4.

CONCLUSIONS

A method has been described which can be used satisfactorily for the quantitative estimation of urinary corticosteroids. The procedure is sufficiently simple to be useful in clinical as well as in research studies.

The extraction is carried out on acidified urine with chloroform. A partition between benzene and water is used and the aqueous fraction thus formed is oxidized with periodic acid. The formaldehyde formed by this reaction is distilled from interfering chromogens and the amount of color formed with chromotropic acid is measured.

The results that have been obtained correspond in order of magnitude to other chemical methods applied to acidified urine.

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HORMONE SECRETION BY HUMAN PLACENTA GROWN IN TISSUE CULTURE

H. L. STEWART, JR., M.D., MACHTELD E. SANO, M.D., AND THADDEUS L. MONTGOMERY, M.D.*

From Temple University Hospital and Medical School and Jefferson Medical College, Philadelphia, Pennsylvania, and The Henry Ford Hospital, Detroit, Mich.

IS THE placenta an organ of internal secretion producing gonadotropins and estrogens during pregnancy? This question has been propounded since 1927 when these hormones were first demonstrated in large amounts in the blood and urine of pregnant women. Many workers have demonstrated the presence of gonadotropins and estrogens in extracts of human placental tissue and it has therefore been accepted that the placenta is an organ of internal secretion and the source of these hormones. Articles bearing on this subject have been voluminous in their theoretical views but have been inconclusive due to lack of direct reasoning and rigid proof.

Renewed interest in the subject has brought forth direct evidence of the secretory function of the placenta. The tissue culture studies of Gey, Seegar, Jones and Hellman, (1, 2) as well as the histochemical presentations of Wislocki, Dempsey and Bennett, (3, 4) and Baker, Hook and Severinghaus (5) are recent and valuable contributions in this field of endocrinology. Our study is presented as further direct evidence of the secretion of the gonadotropins and estrogens by human placental tissue grown in tissue culture.

METHOD OF TISSUE CULTURE

Placental tissue was collected under sterile conditions in the operating room from patients requiring therapeutic termination of pregnancy. The first specimen for tissue culture was obtained by the transvaginal route by cervical dilatation and evacuation of the uterine cavity while in all other cases the abdominal hysterotomy route was used.

Normal appearing placental fragments were selected and placed in a sterile Petri dish with some clotted blood to keep the fragments moist. The tissues were transferred immediately to the tissue culture laboratory.

The tissue culture method used was that originated by Löwenstädt, (6) as suggested to him by Carrel and later revised by Gey (7) with subsequent modifications by Lewis (8). Fragments of placental tissue measuring 2 mm. or slightly less in size were rapidly washed in Tyrode's solution

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* This study was begun in 1941 by the authors at Temple University Hospital and Medical School, and completed by H. L. Stewart, Jr. at the Henry Ford Hospital.

(pH 7.6–7.8). Twenty-eight fragments were implanted in each of four pyrex tubes, 15 × 1.4 cm. The drum in the incubator rotated at a constant speed of one turn in 20 minutes. The incubator was automatically regulated at a temperature of 37.3 degrees centigrade. Several pieces of identical character were selected for histological study. The culture medium consisted of 0.15 ml. of Tyrode's solution, 0.15 ml. of embryonic mouse extract and 0.15 ml. of heparinized chicken plasma (animals less than one year of age). Identical control tubes using mouse spleen or lung tissue were inoculated and at regular intervals this medium was subjected to endocrine assay. The tissue culture procedure was done at room temperature under careful aseptic technique and all solutions and extracts were tested for bacterial contamination. Cell growth was studied under low power in the tubes, while more detailed observations under high power and oil immersion were made in accordance with the Sano-Smith technique (9). At intervals varying from three to seven days, 1.2 cc. of Tyrode's solution was added to the tubes. The tubes were rinsed with this solution by rotating it manually. All washings from these tissue culture tubes were collected and sent to the Laboratory of Endocrinology. As individual pipettes were always used for each step and each solution, the possibility of error and contamination was practically eliminated.

METHOD OF ENDOCRINE ASSAYS

In three placental tissue culture studies, preoperative serum gonadotropin and estrogen assays were made on the blood of the placental donor. On the day of termination of the pregnancy, 50 cc. of blood were obtained by venopuncture. The blood was permitted to clot in a large test tube and was sent directly to the Laboratory of Endocrinology where the clot was broken up and the specimen centrifuged. The serum was used for biological determination of the gonadotropins and estrogens.

Tissue culture washings and tissue culture control specimens were sent directly to the Laboratory of Endocrinology from the Tissue Culture Laboratory. The exact contents of the control specimen were unknown to those doing the hormone titrations until the assays were completed.

All specimens were kept in the icebox without the addition of any preservative and animal injections were begun on the day that the specimens were received. Previous curves for the serum gonadotropins and estrogens during pregnancy had been established in our laboratory on normal pregnant subjects. Suitable dilutions of the sera were made in accordance with the duration of the pregnancy at the time of termination of the gestation. Distilled water was used as the diluent for both sera and tissue culture fluids.

GONADOTROPINS

Adult female isolated rabbits and infantile female mice were used for determinations of the gonadotropin content of the blood and tissue culture washings.

In the standardization of our laboratory animals, we had correlated the relationship of the rabbit to the mouse unit and had found that approximately two and a half mouse units were equivalent to one rabbit unit. This observation permitted us to make a preliminary rapid assay of the gonadotropin content and thereby obtain the approximate amount of hormone within a 48 hour period. Adult female rabbits weighing from 1800 to 2000 grams which had been isolated for 30 days were used. Two rabbits were injected at each dosage level and suitable dilutions of the sera or culture fluid injected over a wide quantitative range. The diluted fluid was injected intravenously in one injection. Forty-eight hours later the ovaries were examined grossly by laparotomy under ether anesthesia. Any ovary showing formation of corpus hemorrhagicum or corpus luteum in one ovary was considered positive. Ovarian sections were made if doubt existed in any case as to the presence of a corpus hemorrhagicum or corpus luteum.

Knowing the approximate titre of the gonadotropins from the preliminary rapid quantitative rabbit determination, we then proceeded with the quantitative assay using infantile female mice, 22 to 25 days of age and weighing from 8 to 10 grams. Injections were made subcutaneously each morning for three days and on the morning of the fifth day the animals were killed by gas asphyxiation. Four to six animals were used at each dosage level.

A final titration was then performed using both infantile female mice and adult isolated female rabbits. As many mice were used as possible with a minimum of 8 to 10 animals for each level. Two rabbits were injected for each level. The presence of corpora hemorrhagica or corpora lutea by histological study of the ovarian section in 50 per cent of the mice and rabbits constituted a positive test.

ESTROGENS

The Fluhmann technique (10) was used for determination of the estrogens in the sera and the tissue culture washings. No dilutions of either fluid were necessary for the amount present was either very minimal or negative Adult spayed female mice weighing 25 grams and approximately 8 weeks of age, 8 days postoophorectomy, were injected subcutaneously twice daily for 3 days with 0.75 cc. for a total of 4.5 cc. The animals were killed on the morning of the fourth day and midvaginal biopsies were made. Histological studies of the vaginal biopsies were made and the amount of estrogens estimated in accordance with the Fluhmann technique.

Six animals were used for each blood serum estimation. The amount of tissue culture fluid limited the number of animals which could be used in each assay, but no less than two animals were used for any one determination.

RESULTS

Histological sections of early placental tissue as well as the more mature placenta of eight and nine months revealed in every case the presence of normal tissue free of any abnormal degeneration or abnormal growth.

The control tissue culture washings showed complete absence of any chorionic gonadotropin and estrogen activity in test animals. Control assays were made at intervals during the study of the placental tissue cultures.

Growth of the adult placenta of eight and nine months duration was unsuccessful on several occasions. Study of these tissue cultures revealed overgrowth of fibroblastic tissue and a complete absence of Langhans and syncytial cells. Endocrine assays were negative for the gonadotropins and estrogens.

PLACENTAL TISSUE CULTURE I

This was placental tissue from a pregnancy of five weeks duration. The termination of pregnancy was by the transvaginal route because of maternal pulmonary tuberculosis. The growth was profuse and three types of cells typically seen in our tissue cultures were present. Cells similar to the syncytial cells appeared to multiply though at no time were actual mitoses noted. Degeneration of these cells appeared early and soon rapid disappearance was evident. Fibroblasts grew relatively well but the Langhans cells with their characteristic clear halo around the nucleus grew most profusely. Fig. 1 is an unstained in vitro culture of the growth seven days after inoculation into the medium showing these cell types. After three weeks the growth rapidly diminished and as shown in Table 1 there was a corresponding diminution of the gonadotropin titre.

Preoperative serum gonadotropin and estrogen assays were not made, for at this time we were interested only in determining whether the placental cells would grow and whether they were active in the production of gonadotropins. Finding that the growth was producing this hormone, our attention was then focused toward correlating the type of cell growth present to the exclusion of any estrogen determinations.

PLACENTAL TISSUE CULTURE II

This was placental tissue from a pregnancy of three months duration. As illustrated in Table 2, preoperative serum gonadotropins showed 20,000 to 24,000 mouse units per 100 cc. of blood while the quantitative Friedman test was positive to 1.65 cc. of 1:20 dilution of the serum. Serum

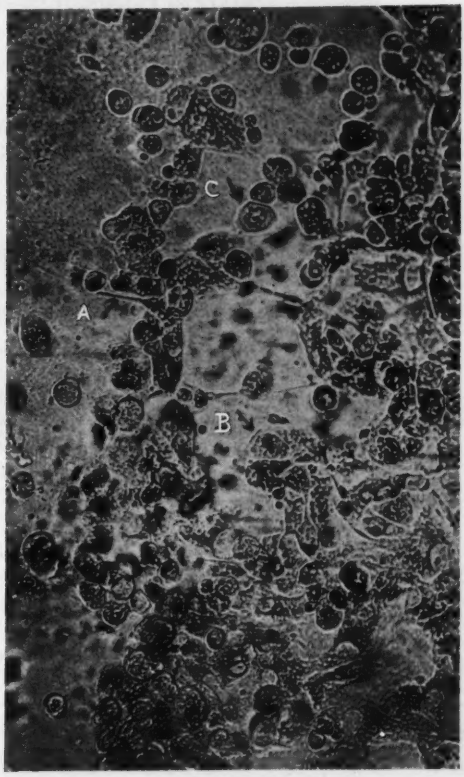

FIG. 1. Unstained tissue culture, seven days after inoculation. ×180. "A"—stromal cell; "B"—syncytium; "C"—Langhans cell.

TABLE 1. PLACENTAL CULTURE I

A.B. Age 35. White. Multipara.
L.M.P. February 1, 1941
C.D.C. November 8, 1941

April 30, 1941—Termination of pregnancy

Quantitative Assay of Tissue Culture Fluid

Specimen	Days Cultured	M.U. per cc. Gonadotropin	Rabbit Gonadotropin	Estrogen
A	7	1.0		
B	14	1.0		
C	19	1.0	No assays	
D	24	0.5		
E	30	negative		

TABLE 2. PLACENTAL TISSUE CULTURE II

T.M. Age 24. White. Multipara.
L.M.P. March 18, 1941.
C.D.C. December 25, 1941.

June 10, 1941—Preoperative serum gonadotropin and estrogen specimen.
Serum gonadotropins:
20,000 to 24,000 M.U. per 100 cc.
Serum estrogens:
6 to 9 M.U. per 100 cc.
Quantitative Friedman Test (serum):
Positive: 1.65 cc. of 1:20 dilution.
Negative: 1.45 cc. of 1:20 dilution.

June 10, 1941—Termination of pregnancy by hysterotomy.

Quantitative Assay of Tissue Culture Fluid

Specimen	Days Cultured	M.U. per cc. Gonadotropin	Rabbit Gonadotropin	Estrogen
A	7	—	—	Negative
B	10	1.5	1.5 cc. positive	—
C	15	1.0	—	Negative
D	20	—	—	Negative
E	27	0.5	—	—
F	31	Follicle stimulation; Uterine growth moderate. No C.H. or C.L.		

estrogen activity showed six to nine mouse units per 100 cc. (Fig. 2). The growth was not particularly good and the Langhans cells visualized early as growing, soon degenerated after two and one-half to three weeks. Hormone assays for the gonadotropins showed a parallel decrease in activity.

PLACENTAL TISSUE CULTURE III

Table 3 shows in graphic form the assays of a placental tissue culture growth of approximately seven weeks duration. Preoperative serum gonadotropins were approximately 3500 mouse units per 100 cc. Serum estrogens were not demonstrable. Injections of 1.35 cc. of 1:40 dilution of the donor serum gave a positive Friedman test.

Growth was profuse and the most abundant we observed in any of our cultures. Numerous well balanced mitoses were observed. Langhans cells predominated throughout and became more abundant as the tissue grew.

Figure 3 shows the Langhans cells with the clear halo around the nucleus and several mitoses. By the Sano-Smith method it is possible to stain these cells and mount the slides without manipulating or disturbing the growth.

Figure 4 portrays the cells at the peak of their growth with evidence of

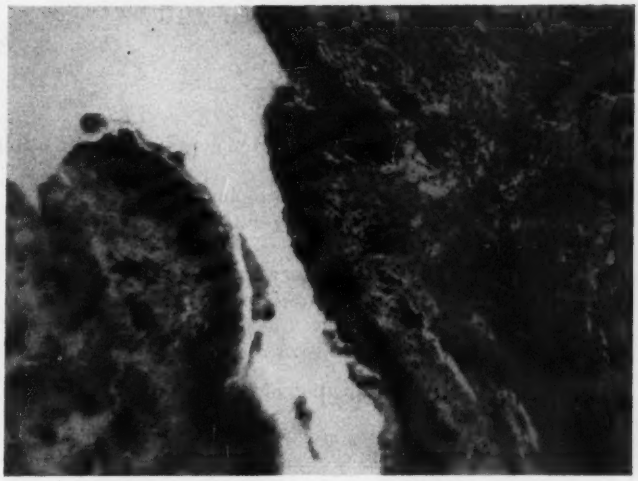

FIG. 2. Vaginal mucosa of mouse showing reaction 3 in the mucification test for estrogen; approximately 6 mouse units per 100 cc. of blood.

degeneration of a few. This photograph was taken one month after they had been inoculated into the medium. At this time there was a rise in the gonadotropin titre. Six weeks later they were still healthy in appearance but not actively growing as noted by the presence of only a rare mitosis.

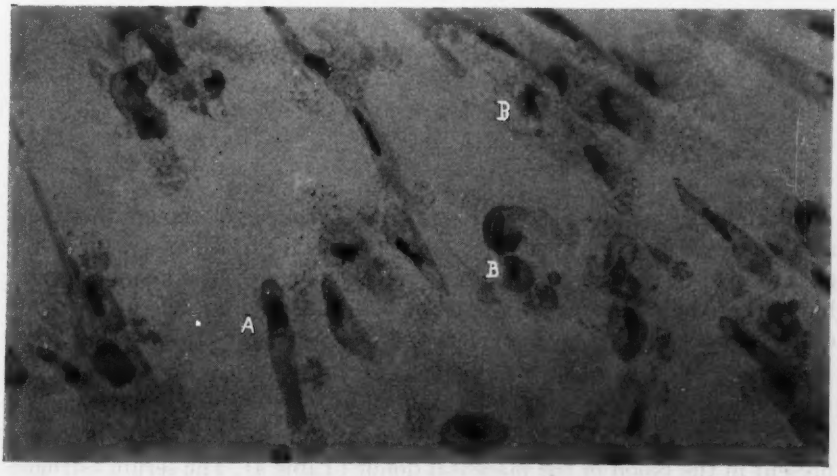

FIG. 3. Twelve days growth. "A"—syncytium; "B"—Langhans cell. ×300. Toluidine blue stain.

TABLE 3. PLACENTAL CULTURE III

D.B. Age 30. White. Multipara.
L.M.P. August 26, 1941.
C.D.C. June 2, 1942.

October 24, 1941—Preoperative serum gonadotropin and estrogen specimen.
Serum gonadotropins:
3500 M.U. per 100 cc.
Serum estrogens:
None demonstrable.
Quantitative Friedman Test (serum):
Positive: 1.35 cc. of 1:40 dilution.
Negative: 1.20 cc. of 1:40 dilution.

October 24, 1941—Termination of pregnancy.

Quantitative Assay of Tissue Culture Fluid

Specimen	Days Cultured	M.U. per cc. Gonadotropin	Rabbit Gonadotropin	Estrogen
A	5	—	—	Negative
B	10	55	0.5 cc. 1:40	Negative
C	13	50	1.0 cc. 1:40	Negative
D	19	40	1.5 cc. 1:40	—
E	22	—	—	Negative
F	25	22	1.0 cc. 1:20	Negative
G	31	26	1.0 cc. 1:20	—
H	35	—	—	Negative
I	40	20	1.0 cc. 1:20	—
J	45	—	2.0 cc. 1:20	Negative
K	50	5	—	Negative
Study discontinued.				

In this study repeated estrogen assays were made even though there was an absence of other cell types including syncytial cells. All assays for estrogens were negative and Figure 5 is a typical example of the many negative biopsies of the mouse vaginal mucosa. Difficulty in growth of the syncytial cells was constantly in evidence.

PLACENTAL TISSUE CULTURE IV

Prior to termination of pregnancy, large amounts of gonadotropins were present in the blood of the placental donor (Table 4). The serum estrogen was approximately 12 mouse units per 100 cc.

The tissue culture growth was quite abundant although less than in

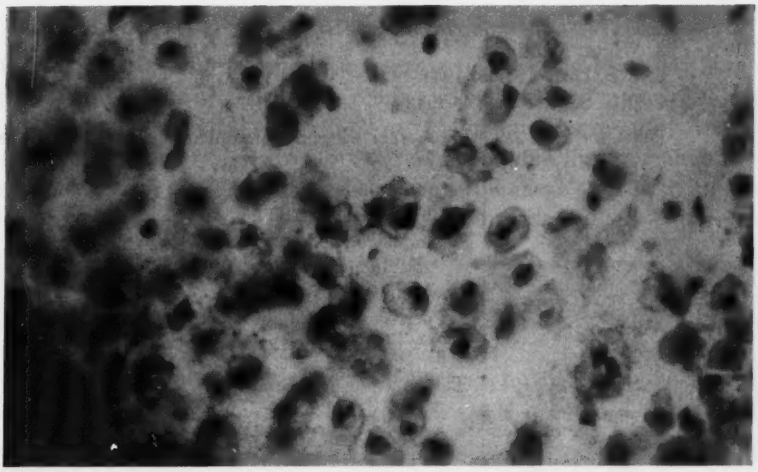

FIG. 4. Peak of hormone production. Some of the Langhans cells are undergoing degeneration. Mostly Langhans cells. ×300. Toluidine blue.

placental Tissue Culture IV. Fewer mitoses were present. The cytoplasm of the Langhans cells was slightly more granular (Fig. 6). The gonadotropin titre was not as high. Degeneration of the cells had appeared to set in about the third week and little hormone production was expected. However, titrations of the washings proved the contrary. The mouse ovaries

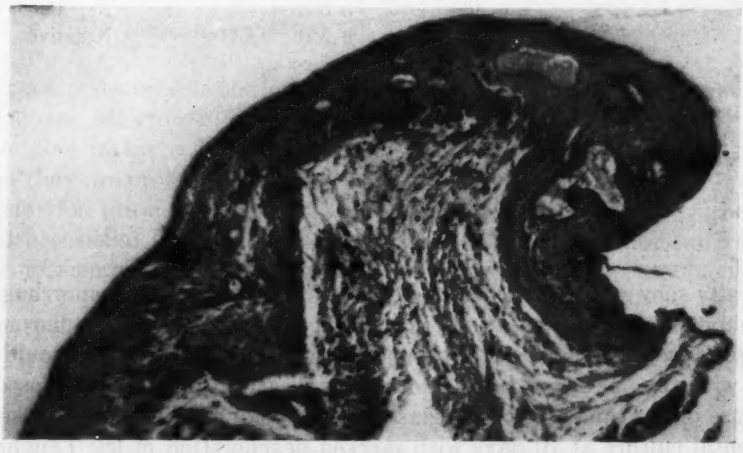

FIG. 5. Vaginal mucosa of mouse showing no estrogenic response. Mucosa shows two layers of low cuboidal epithelium.

TABLE 4. PLACENTAL CULTURE IV

D.R. Age 23. White. Multipara.
L.M.P. December 9, 1941.
C.D.C. September 16, 1942.

February 20, 1942—Preoperative serum gonadotropin and estrogen specimen.
Serum gonadotropins:
4000 M.U. per 100 cc.
Serum estrogens:
12 M.U. per 100 cc.
Quantitative Friedman Test (serum):
Positive: 2.25 cc. of 1:40.
Negative 1.50 cc. of 1:40.

February 21, 1942—Termination of pregnancy.

Quantitative Assay of Tissue Culture Fluid

Specimen	Days Cultured	M.U. per cc. Gonadotropin	Rabbit Gonadotropin	Estrogen
A	5	40	2.5 cc. 1:40	
B	10			Negative
C	13	35	3.0 cc. 1:40	Negative
D	18		3.0 cc. 1:20	Negative
E	23	20	2.0 cc. 1:20	
F	28	12	2.0 cc. 1:10	Negative
G	33	10	2.0 cc. 1:10	
H	38	8	3.0 cc. 1:10	Negative
I	44	4	0.5 cc.	Negative
J	48	6	0.5 cc.	
K	55	4	0.5 cc.	
L	61	2	0.5 cc. Negative	
M	70	1	1.0 cc. Negative	
N	75 Follicle stimulation. Uterine growth marked. No C.H. or C.L.		1.0 cc. Negative	

typically showed the presence of corpora lutea and corpora hemorrhagica two months after implantation (Fig. 7). It was believed that the hormone was produced by fragments of tissue deeper in the medium which could be seen with the naked eye but could not be observed under the microscope because of the thickness of the glass and the medium. Figure 8 shows the placental culture at 70 days with marked degeneration of the Langhans cells.

Syncytial cells failed to grow well and all estrogen assays were negative.

DISCUSSION

Our placental tissue culture studies revealed that in the early stages of growth three types of cells could readily identified:—stromal cells with a round nucleus, syncytial cells which are oblong and have an oval nucleus, and Langhans cells which are more or less round and have a round nucleus surrounded by a pale halo (Fig. 1). After a few days of growth the latter cells predominated. Soon a direct relationship was noted between any

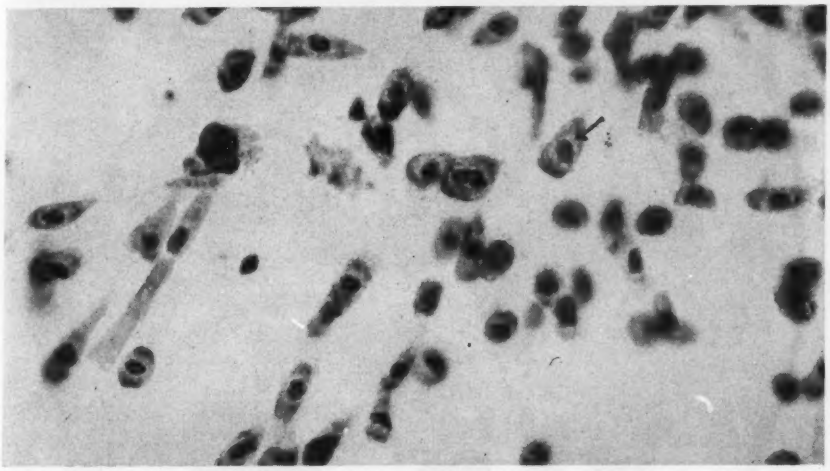

FIG. 6. Four days growth, mostly Langhans cells, in three weeks old placental tissue. Note the clear halo around the nucleus (arrow).

increase or decrease in the hormone titrations for the gonadotropins and the Langhans cell growth. This suggested to us the important role that these cells play in the secretion of the gonadotropic hormone and we believe that they are probably responsible for the great amounts of this hormone production during pregnancy. Baker, Hook and Severinghaus (5) believe that occasional Langhans cells persist beneath the syncytium until term. It is reasonable to assume that the relatively lower level of the blood and urinary levels of gonadotropins in later pregnancy fall in direct correlation with the fewer number of Langhans cells.

Our results are in support of those of Gey and his coworkers (1, 2). They showed that placental cells maintained in continuous culture for as long as six months produced gonadotropins. The cells apparently responsible for this activity were the Langhans cells.

It is interesting to note that our preoperative serum assays for the estrogenic hormone were small in amounts until the third month of pregnancy.

In two cases, 3 and 4, small amounts were present showing some increased production at the time of termination of the pregnancy. Wislocki and Dempsey (3, 4) as well as Baker, Hook and Severinghaus (5) believe that the syncytial cells are the source of estrogen formation in the placenta. In our tissue cultures, syncytial cells were noted in the early growth stages but quickly disappeared as the Langhans cells became the predominant cell to the

FIG. 7. Infantile mouse ovary showing formation of corpora hemorrhagicum and luteum. Reaction obtained by injection of 0.5 cc. tissue culture washing 60 days after placental tissue implanted.

exclusion of the syncytial cells. In placental tissue culture assays of III and IV, many estrogen determinations were made without any evidence of its presence. Yet our negative results, we believe, are inconclusive and not in favor of or against the secretion of estrogens by the placenta. Since so little increase of estrogen was present in the blood of the patient prior to termination of pregnancy and since the syncytial cells did not grow in tissue

culture, demonstrable amounts could not be expected from our tissue cultures on the basis of the present viewpoint.

Our curves of estrogen in the blood and urine of normal pregnant women show a gradual rise from the third month to the highest peak just prior to parturition. Because of this we attempted on several occasions to grow the more mature placenta of eight and nine months. The growth was un-

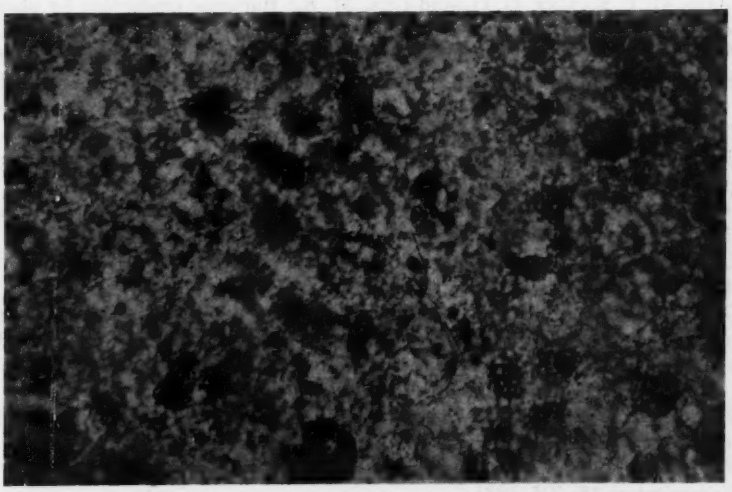

FIG. 8. After peak of hormone production there is marked degeneration of the cells. ×300. Toluidine blue.

successful for fibroblasts grew in profusion without demonstrable syncytial or Langhans cell growth. It is interesting that the trophoblastic tissue of early pregnancy grows so readily in comparison with the more mature or adult placenta.

CONCLUSIONS

1. Human placental cells of approximately three months of age, grown in continuous tissue culture in vitro, showed the production of the gonadotropic hormone as assayed quantitatively in rabbits and infantile mice.
2. A direct correlation was noted between the growth of the Langhans cells and the production of the gonadotropic hormone.
3. Control tissue cultures in which fibroblasts were grown produced no hormone response in the test animals.
4. Attempts to grow the adult placenta of eight and nine months were unsuccessful and resulted only in overgrowth of fibroblastic tissue.

5. Syncytial cells did not grow well in tissue culture.
6. Evidence concerning the production of estrogen by the placental cells was inconclusive.

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ENDOCRINE REVIEWS

There has been a great demand from our members for good review articles in the Journal of Clinical Endocrinology. It was felt that these articles would be of value both to endocrinologists and to physicians in other fields of medicine who wish to keep themselves informed about advances in endocrinology. The excellent review by Dr. Harold L. Mason of the Mayo Clinic on Steroid Nomenclature is the first of a series of such articles. Many of our members have already agreed to prepare reviews for the journal. These will appear from time to time as they become available.

WILLARD O. THOMPSON,
Managing Editor.

STEROID NOMENCLATURE

HAROLD L. MASON, Ph.D.
Division of Biochemistry, Mayo Foundation, Rochester, Minnesota

INTRODUCTION

IT IS the purpose of this paper to consider the nomenclature of the steroid hormones and some of their important derivatives. It is intended for those who have occasion to deal with steroids but who do not have an intimate acquaintance with their chemistry or with the principles by which they are named. The complexities of some of the names and lack of agreement among chemists about some details of terminology may well serve to confuse the uninitiated. Almost any compound may be named in several different ways. The chemist often names a compound in a manner that serves to emphasize a certain feature or features of that compound. In this paper the names that are used most commonly in this country will be emphasized and variations in these names will be given. It is purposed only to interpret steroid nomenclature, not to advocate any particular system. However, some preferences will be stressed in the interests of good chemical usage.

THE STEROID RING SYSTEM

The fundamental ring system of the sterols, bile acids, sex hormones and adrenal hormones, the latter three being grouped under the term "steroids"

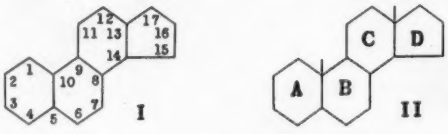

FIG. 1

is the perhydrocyclopentenophenanthrene ring system (Fig. 1, I). The system[1] used for numbering the carbon atoms of the steroids is also shown in formula I. This is a saturated ring system.[2] Each juncture of two or

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[1] It should be pointed out that the cyclopenteno ring (the ring with five carbon atoms) may be attached to phenanthrene in several different positions with the formation of a number of different cyclopentenophenanthrenes. Also, the steroids have a unique numbering system which is not related to the system that has been adopted for phenanthrene and its derivatives.

[2] The name "perhydrocyclopentenophenanthrene" may seem to disagree with the

more lines in I and II represents one carbon atom. Each carbon atom, except those common to two rings, bears two hydrogen atoms. Ordinarily these hydrogen atoms are not written in the structural formula. It is understood that those bonds of the carbon atoms which are not shown are combined with hydrogen. A hydrogen atom attached to a carbon atom which is common to two rings may be written in to show the stereochemical structure at this point. The estrogens have a methyl group (CH_3) attached at position 13. Most of the other steroids (II) have methyl groups (indicated by the short vertical lines in II) attached at carbons numbered 10 and 13 (C-10 and C-13). The methyl groups are known as angular methyl groups because they occur in the angle formed by the juncture of two rings.

SUBSTITUTION IN THE STEROID RING SYSTEM

In conformity with the rules of organic nomenclature, many steroids are named as derivatives of a few hydrocarbons such as cholane, etiocholane, pregnane, and so forth. Others, such as androsterone and progesterone, have received trivial[3] names which are commonly used. The ending "-ane" is reserved for saturated hydrocarbons. The ending "-ene" indicates a hydrocarbon with a double bond; "-diene," "-triene," "-tetraene" indicate the presence of two, three, four double bonds, and so on. Substitution of oxygen for hydrogen in the hydrocarbon is shown by addition of "-ol" or "-one" to the name of the hydrocarbon. The suffix "-ol" represents an alcohol group (hydroxyl or OH) and "-one," a ketone group (C=O, also called a carbonyl group). Examples: pregnane, pregnanol, pregnanone (the final *e* of "-ane" and "-ene" is dropped when followed by another vowel), pregnanediol, pregnanedione; pregnene, pregnenol, pregnenediol. Alternatively these substituents may be indicated by the prefixes "hydroxy-" and "keto-" (or "oxo-") respectively. Pregnanol would thus be hydroxypregnane; pregnanone would be ketopregnane (or oxopregnane), and pregnanediol would be dihydroxypregnane. These prefixes and suffixes, and some others, and their meanings, are listed in Table 1 for convenient reference.

If a compound contains both hydroxyl (alcohol) and ketone groups the order in which the prefixes or suffixes appear in the name is largely a matter of choice. Usually the one that indicates a hydroxyl group (or groups)

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naming of saturated hydrocarbons which is discussed in the next paragraph. However, the prefix "perhydro-" means that cyclopentenophenanthrene (an unsaturated hydrocarbon) has been saturated with hydrogen. There is no simple name ending in "-ane" for this saturated hydrocarbon. In a similar manner the saturated hydrocarbon ethane could be named "dihydroethylene."

[3] "Trivial" is used in the sense of common or ordinary.

TABLE 1. MEANINGS OF SUFFIXES AND PREFIXES USED IN NAMING THE STEROID HORMONES

Suffix	Meaning
-ane	Saturated hydrocarbon. Cannot take up more hydrogen
-ene	Unsaturated hydrocarbon; has double bonds. Can take up more hydrogen
-ol	Alcohol; hydroxyl group; OH
-one	Ketone; carbonyl group; C=O
Prefix	
hydroxy-	Hydroxyl group; OH
keto-	Ketone; carbonyl group; C=O
oxo-	Same as "keto"
allo-	Other; meaning one of two isomers
cis-	Refers to arrangement of two groups on the same side of a molecule
trans-	Refers to arrangement of two groups, one of which is on the opposite side of a molecule from the other
etio-	Refers to the final degradation product of a more complex molecule which still retains the essential chemical character of the original molecule
iso-	Refers to isomers
epi-	Refers to isomers which differ only in the spatial arrangement of groups about one carbon atom
desoxy-	An oxygen atom has been lost
dehydro-	Two atoms of hydrogen have been removed

precedes the one that indicates a ketone group (or groups). Thus "pregnanolone" is preferred to "pregnanonol" and in the alternative method of naming, "hydroxyketopregnane" ("hydroxyoxopregnane") is preferred to "ketohydroxypregnane."

Each carbon atom of a hydrocarbon is assigned a number. When a hydrogen atom (or atoms) attached to a carbon atom is substituted by another

atom or group of atoms, the number of that carbon atom is placed before the prefix or suffix which designates the nature of the substituent. Thus, pregnan-3-ol or 3-hydroxypregnane; pregnane-3,20-diol or 3,20-dihydroxypregnane. In some of the literature the numbers follow the substituents, as in pregnanediol-3,20, pregnanol-3-one-20. However, it is preferable to follow the usage of Chemical Abstracts and the Journal of the American Chemical Society and to place the number before the substituent.

Some authors arrange the suffixes and their numbers in such order that the lowest number comes first. They would write "pregnan-3-ol-20-one" but "pregnan-3-on-20-ol" instead of "pregnan-20-ol-3-one," and "11-dehydro-17-hydroxycorticosterone" rather than "17-hydroxy-11-dehydrocorticosterone." This method is used by Selye (1).

DOUBLE BONDS

A double bond and its position are often indicated by the symbol Δ used as a prefix and with a superscript which shows the position of the double bond. Examples: Δ^5-androstene, Δ^4-pregnene-3,20-dione. It is understood that the bond originates at the carbon atom shown by the superscript, and terminates at the carbon atom with the next higher number. If there is any doubt, numbers are given for both ends of the double bond. For example, a bond originating at C-8 may terminate at C-9 or at C-14. In this instance the position of the double bond is shown by $\Delta^{8,9}$ or $\Delta^{8,14}$ as the case may be. Use of Δ^8 would be ambiguous. The suffix "-ene" is always used in addition to Δ. If more than one double bond is present the positions of both are shown and two double bonds are indicated in the name. Examples: $\Delta^{3,5}$-androstadiene-17-one, $\Delta^{5,16}$-pregnadiene. Multiple double bonds are shown in similar fashion. Although it might appear that some ambiguity would result from the use of two numbers in the superscript to indicate one or two double bonds, a brief consideration of the numbers and the suffix will solve the difficulty. In the examples given, it would not be possible to have a double bond between carbon atom 3 and carbon atom 5 and the suffix "-diene" shows that two double bonds are present. Use of $\Delta^{8,9}$ or $\Delta^{8,14}$ and the suffix "-ene" definitely limits the compound to one double bond between the carbon atoms indicated. Use of $\Delta^{8,14}$ and the suffix "-diene" would indicate double bonds between carbon atoms 8 and 9 and between carbon atoms 14 and 15.

There is no general agreement about the superscripts used with Δ. The system explained here is used generally in this country. More complex situations than those illustrated may arise and necessitate some modification of this simple usage. The estrogens are a case in point, as will be shown later.

It is not essential to use Δ. The compounds in the examples given may

be named 5-androstene, 4-pregnene-3,20-dione, 3,5-androstadiene-17-one, and 5,16-pregnadiene. In "androstadiene" and "pregnadiene" an *a* has been added between the root and the suffix for euphony.

ISOMERISM INVOLVING CARBON ATOMS COMMON TO TWO RINGS

When saturated rings are fused in the manner shown in Fig. 2 (III), that is, with two carbon atoms common to both rings, two isomers are

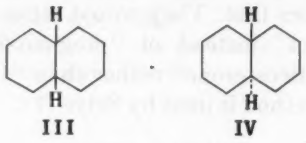


FIG. 2

possible depending on whether the hydrogen atoms attached at the junctures of the rings are on the same side (cis as in III) or on opposite sides (trans as in IV) of the plane of the rings. In the steroid ring system this type of isomerism may involve carbon atoms 5 and 10, 8 and 9, and 13 and 14. The relative arrangement of the hydrogen atom or methyl group on each of these atoms may determine two isomers for each carbon atom. Therefore the number of possible isomers is 2^6 or 64. Fortunately, this type

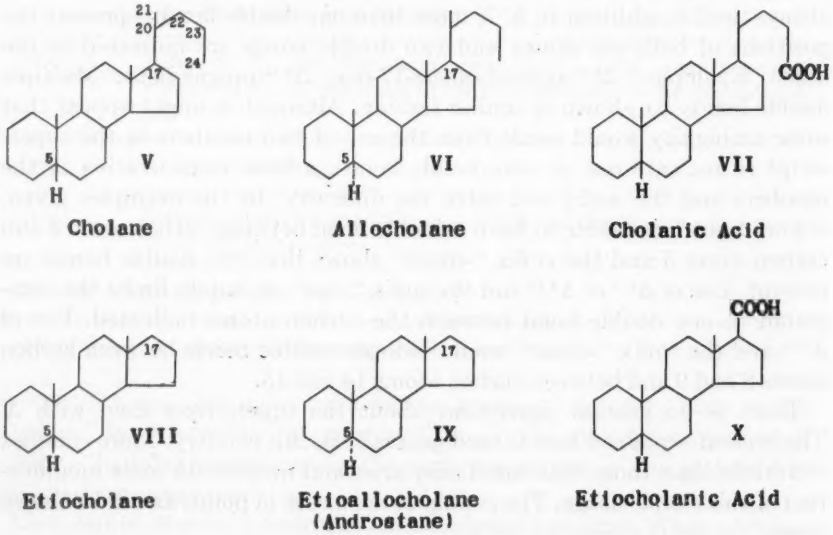


FIG. 3

of isomerism is limited to C-5 in the commonly occurring steroids related to hormones.

In the hydrocarbon cholane (Fig. 3, V), which has the carbon skeleton of the bile acids, the methyl group attached to C-10 and the hydrogen atom at C-5 are considered to be on the same side of the ring system (cis relation). The hydrocarbon with the hydrogen atom at C-5 having the opposite (trans) arrangement with relation to the methyl group at C-10 is called allocholane (VI). In formula V, the bond holding the hydrogen atom at C-5 is written with a solid line to show that the hydrogen atom is on the same side of the ring system as the methyl group at C-10 (above the plane

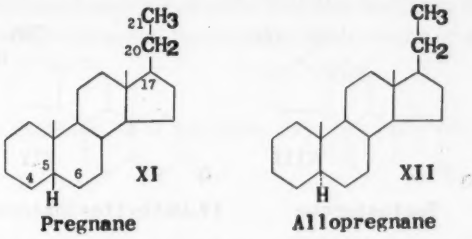


FIG. 4

of the paper). In formula VI, the bond at C-5 is written with a broken line to show that the hydrogen atom is on the opposite side of the ring system from the methyl group at C-10. In the naturally occurring steroids, the methyl groups at C-10 and C-13 are considered always to have the same relation to the rest of the molecule, that is, above the plane of the molecule as written, and the bond is shown as a solid line.

If the chain of carbon atoms attached at C-17 in cholane and allocholane is removed, the corresponding hydrocarbons are etiocholane (VIII) and etioallocholane (IX). Etioallocholane is called more commonly "androstane." The prefix "etio-," as used in steroid chemistry, indicates that the compound is the simplest one that can be obtained by degradation of the parent substance and still retain the steroid ring system and the fundamental chemical characteristics of the parent substance. Thus, etiocholane is the steroid ring system with the two methyl groups whereas etiocholanic acid (X) has a carboxyl group at C-17. Degradation of etiocholanic acid by one carbon atom more would not give an analogue of cholanic acid (VII) but a neutral compound (etiocholane).

The hormones and related compounds may be named as derivatives of etiocholane or of androstane (etioallocholane). This system is used by Selye. It is often more convenient, however, to name some compounds as derivatives of pregnane (Fig. 4, XI) and allopregnane (XII), which are

17-ethyl derivatives of etiocholane and etioallocholane (androstane), respectively.

It will be noted that in pregnane (or allopregnane) the numbers jump from 17, the last carbon atom in the ring system, to 20, the first carbon atom in the side chain and that C-17 and C-20 are joined together. The missing numbers, 19 and 18, are assigned to the two methyl groups attached at C-10 and C-13. These numbers have not been included since there is some disagreement as to which methyl group should receive which number. It might be pointed out, however, that it would seem to be logical to assign the number 18 to the methyl group at C-13 and 19 to the methyl

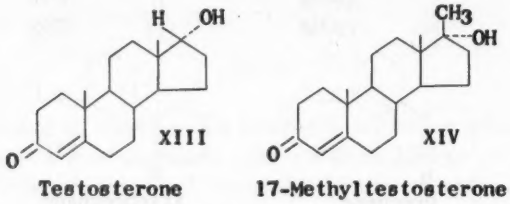


FIG. 5

group at C-10 since the methyl group at C-10 is missing in the estrogens. If the methyl group at C-10 is numbered 18 and that at C-13 is numbered 19, then there would be a gap in the numbering system of the estrogens.

A double bond at 4-5, as in testosterone (Fig. 5, XIII) or progesterone (Fig. 8, XX), or at 5-6, as in dehydroisoandrosterone (Fig. 7, XVII), eliminates the possibility of isomerism at C-5 and the simpler name may be used with the ending "-ene"; examples, androstene, pregnene.

(α) AND (β) CONFIGURATIONS

Other important points at which isomerism may occur are at C-3, C-11, and C-20. At C-3 and C-11 the two valences of the carbon atom that are not attached to other carbon atoms are so arranged that one extends above and the other below the plane of the ring system. Substitution of one of the hydrogen atoms with OH, for example, offers two possibilities. The OH may be above or below the plane of the rings and thus two different compounds are possible. Since both angular methyl groups are considered to be on the same side of the ring system and, by convention, to be above the plane of the ring system as it is ordinarily written in the plane projection, it is customary to refer the configuration of all substituents to one or the other methyl group. Those substituents that are on the same side of the ring system as the methyl groups have the (β) configuration and those

on the opposite side the (α) configuration. In accord with the convention used to indicate the position of the hydrogen atom at C-5 the (α) configuration is ordinarily shown by a broken line for the bond connecting the substituent to the carbon atom in question while the (β) configuration is indicated by use of a solid line for the bond.

The use of a solid bond to indicate the (β) configuration is somewhat ambiguous, since a solid line is ordinarily used to indicate a bond without any implications as to configuration. In many cases the configuration is unknown. Therefore care must be used in the interpretation of formulas in which (α) and (β) positions are indicated by dotted and solid lines, respectively. A solid bond may mean only that the configuration is unknown. This difficulty could be avoided by showing both atoms or groups attached to the carbon in question. For example,

may be ambiguous but

could mean only that the configuration is not specified (and therefore presumally unknown) since the H and OH cannot both be in the (β) position.

In the name of a compound the configuration of a substituent is shown by writing α or β in parentheses after the number of the carbon atom involved. Example: Δ^5-androstene-3(β),17(α)-diol. "Trans" and "cis" are sometimes used interchangeably with (α) and (β), respectively, since substituents in the (α) position are on the opposite side of the molecule from the methyl groups and those in the (β) position are on the same side. However, this usage is not always consistent. For example, dehydroisoandrosterone has also received the name trans-dehydroandrosterone but the OH at C-3 (to which "trans" refers) has the (β) configuration. In this instance "trans" is not used with reference to the methyl group at C-10.

Epimers are isomers which differ only in the steric arrangement of the groups about one carbon atom. The prefix "epi-" is used to indicate such a relation. Androsterone and isoandrosterone are epimers and the latter could be called "epiandrosterone." In names of steroids the prefix "iso-" often is used interchangeably with "epi-" as in the case of isoandrosterone. The carbon atom involved is indicated in the usual way if more than one carbon atom may be involved. Thus, if the 12-hydroxyl group of desoxycholic acid is caused to assume the configuration opposite to the natural one, the resulting compound would be 12-epidesoxycholic acid or 12-iso-

desoxycholic acid. It should be added, however, that the prefix "iso-" may be used to indicate any type of isomerism in organic chemistry.

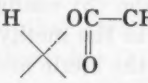
With reference to the steroids under consideration the prefix "allo-" (Greek allos, other) usually refers specifically to isomerism at C-5. This type of isomerism was illustrated by cholane (Fig. 3, V) and allocholane (VI) and their derivatives. Cholane, etiocholane (VIII) and pregnane (Fig. 4, XI) are considered to have the "normal" arrangement at C-5. Compounds with the opposite arrangement at C-5 are allo compounds such as etioallocholane (androstane).

The terms "epi-," "iso-," and "allo-" have other connotations in organic chemistry and even with reference to steroids (for example, "allocholesterol" which has no hydrogen atom at C-5). However, they have the specific meanings indicated in connection with the compounds discussed in this paper.

With these principles in view we may proceed to consider specific applications.

ANDROGENS AND RELATED SUBSTANCES

Testosterone (Fig. 5, XIII) is the trivial name of the compound generally considered to be the androgenic hormone produced by the testis. A systematic name would be Δ^4-androsten-17(α)-ol-3-one or Δ^4-etiocholen-17(α)-ol-3-one. With a double bond at 4-5 androstene and etiocholene are identical. Given the name, one can write the structural formula by drawing the steroid ring system with the angular methyl groups, placing a ketone group at C-3, a double bond between carbon atoms 4 and 5 and a hydroxyl group attached to C-17 with a broken line. The group involving C-3 to C-5 is generally called an α,β-unsaturated ketone group and is characteristic of all of the most active steroid hormones with the exception of the estrogens. Change of this group in any detail reduces or abolishes the specific activity of the hormone in question.

Testosterone propionate usually is used for therapy. In this compound a molecule of propionic acid has been combined with the 17-OH to form the ester group.  . Since there is only one hydroxyl group to enter into ester formation it is not necessary to be more specific but if there were more than one hydroxyl group present it would be necessary to specify with a number which hydroxyl group is involved.

An isomer of testosterone is known which has the 17-OH in the (β) position. It is called cis-testosterone since a 17-OH in the (β) position has the cis relation to the methyl group at C-13, that is, these two groups are on the same side of the molecule. Another name for cis-testosterone is Δ^4-androsten-17(β)-ol-3-one.

The synthetic compound, methyltestosterone (XIV), has a methyl group at position 17 as well as a hydroxyl group and would properly be named "17-methyltestosterone" to indicate the position of the methyl group. A systematic name would be "17-methyl-Δ^4-androsten-17(α)-ol-3-one." Other androgens have been isolated from adrenal extracts: Δ^4-androstene-3,17-dione, 11(β)-hydroxyisoandrosterone (androstane-3(β), 11(β)-diol-17-one) and adrenosterone (Δ^4-androstene-3,11,17-trione). It is possible that these substances are artefacts produced during the process of isolation but there is no evidence on which to base a decision.

The known metabolites of testosterone are included in the group of

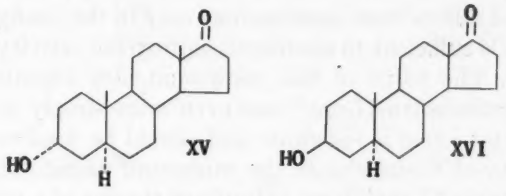


FIG. 6

compounds known as the "neutral" (to distinguish them from estrogenic phenols) 17-ketosteroids. These compounds are so named because of the presence of a ketone group at C-17. All of the common ones also have a hydroxyl group at C-3. The trivial name "androsterone" (Fig. 6, XV) has been given to androstan-3(α)-ol-17-one (or etioallocholan-3(α)-ol-17-one). Its isomer with the 3-hydroxyl group having the (β) configuration but with

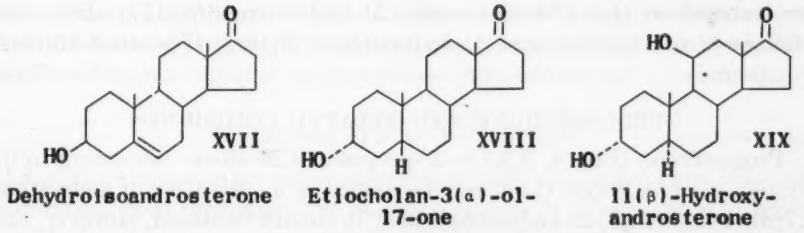


FIG. 7

the rest of the molecule identical is isoandrosterone, or androstan-3(β)-ol-17-one (XVI). If a double bond is introduced in isoandrosterone by loss of a hydrogen atom from C-5 and one from C-6 (dehydrogenation) the resulting compound is dehydroisoandrosterone (more specifically 5-dehy-

droisoandrosterone) or Δ^5-androsten-3(β)-ol-17-one (Fig. 7, XVII). Dehydroisoandrosterone sometimes is named "trans-dehydroandrosterone" (*t*-dehydroandrosterone) but the former term is preferred. This substance and isoandrosterone comprise the major portion of the (β) fraction (so called because of the (β) configuration of the 3-hydroxyl group) of the 17-ketosteroids. The amount of the former compound is greatly increased in most patients who have adrenal cortical tumors and therefore a determination of the (β) fraction as well as the total amount of 17-ketosteroids may be helpful for differentiation between the presence of a tumor and cortical hyperplasia.

Another isomer of androsterone is etiocholan-3(α)-ol-17-one (XVIII). This compound differs from androsterone only in the configuration at C-5 The difference is sufficient to abolish all androgenic activity in etiocholan-3(α)-ol-17-one. The name of this compound also sometimes is written "3(α)-hydroxyetiocholan-17-one" and even more simply as "etiocholanolone." The latter name is indefinite and should be used only when there is no possibility of mistake as to the compound meant. The name "3(α)-hydroxyetiocholan-17-one" is an example of the use of a prefix to indicate one substituent and the use of a suffix to indicate another substituent.

Another 17-ketosteroid found in normal urine is 11(β)-hydroxyandrosterone or androstane-3(α),11(β)-diol-17-one (XIX). This substance is generally considered to be a metabolite of adrenocortical steroids, since it has the oxygen atom at C-11 which is characteristic of certain cortical hormones. Some of the other 17-ketosteroids, particularly dehydroisoandrosterone, and part of the androsterone and etiocholanolone, are derived from adrenal steroids but their relation to the known adrenal compounds is obscure.

Urine also may contain alcoholic steroids without a ketone group which are related to the 17-ketosteroids. Δ^5-Androstene-3(β),17(α)-diol, etiocholane-3(α),17(α)-diol, and Δ^5-androstene-3(β),16(β),17(α)-triol are such compounds.

PROGESTERONE AND RELATED COMPOUNDS

Progesterone (Fig. 8, XX) is Δ^4-pregnene-3,20-dione. According to the system used by Selye, (1) it may be named as a derivative of androstene: 17(β)-[1-ketoethyl]-Δ^4-androsten-3-one. It should be noted, however, that the 1-ketoethyl side chain is now known to be attached in the (β) position rather than in the (α) position as given by Selye.

The chief metabolite of progesterone is pregnane-3(α),20(α)-diol (XXI), which occurs in relatively large amounts in the urine of pregnancy. The name is often shortened to "pregnanediol." Unless otherwise indicated, this name may be interpreted to mean the 3(α),20(α)-diol. The other three

possible isomers are known and pregnane-3(β),20(α)-diol, which occurs in minor quantities, probably also is a metabolite of progesterone.

The designation '20(α)" does not have the same structural significance as, for example 3(α) or 17(α) since C-20 is in the side chain. However, the bond between C-20 and the OH is often shown as a broken line to indicate the (α) compound or as a solid line to indicate the (β) compound. In this case (α) and (β) serve to identify certain compounds but the precise relation of the substituent at C-20 to the rest of the molecule is uncertain.

The name "pregnanediol" often is spelled "pregnandiol," without the *e* of the "-ane" suffix, and is pronounced "preg-nan'-dee-ol." According to

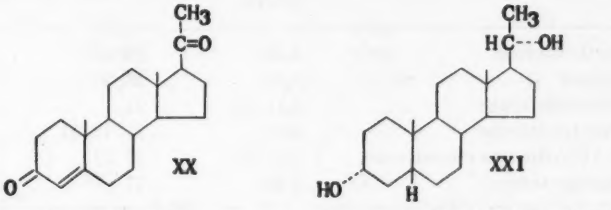


FIG. 8

the principles discussed at the beginning of this paper, this spelling and pronunciation obviously are not correct. "Prĕg'-nāne-dī'-ōl" indicates immediately the chemical nature of the compound whether written or spoken.

Pregnan-3(α)-ol-20-one also is a metabolite of progesterone. Recently it has been found to be an appreciable fraction of the sodium pregnanediol glucuronidate of pregnancy urine as ordinarily isolated (2).

Δ^5-Pregnen-3(β)-ol-20-one, usually named simply "pregnenolone," has been isolated from hog testes (3). It is reported to have a favorable effect on endurance and fatigue (4) and to be effective in maintaining testicular weight and spermatogenesis in hypophysectomized rats (5).

ADRENOCORTICAL HORMONES

All of the characteristic adrenocortical steroids that have been isolated are derivatives of pregnane. The chemical details of those known to have physiologic activity are summarized in Table 2. Progesterone and estrone have been isolated from adrenal extracts but they are not included in Table 2, since they are also ovarian products. Androgenic substances with the same number of carbon atoms as androsterone also have been isolated from adrenal extracts. They are considered with the other androgens. 17-Hydroxyprogesterone is included with the adrenal hormones because of its

close structural relationship, although its function is unknown beyond the fact that it has androgenic properties (6).

The first adrenocortical hormone to receive a trivial name was corticosterone and the other hormones have been named in systematic fashion as derivatives of corticosterone. Referring to Table 2, "11-desoxy-" means

TABLE 2. ADRENOCORTICAL HORMONES

Ordinary name	Chemical details		
	C=O	OH	Double bond
11-Desoxycorticosterone	3,20	21	4–5
Corticosterone	3,20	11, 21	4–5
11-Dehydrocorticosterone	3,11, 20	21	4–5
17-Hydroxycorticosterone	3,20	11, 17, 21	4–5
11-Dehydro-17-hydroxycorticosterone	3,11, 20	17, 21	4–5
17-Hydroxyprogesterone	3,20	17	4–5

that an oxygen atom of corticosterone (Fig. 9, XXII and XXIII) has been removed and replaced by hydrogen at C-11. "11-Dehydro-" means that two hydrogen atoms have been removed at C-11. Since corticosterone (XXII) has OH at C-11, removal of two hydrogen atoms converts the >CHOH group to a >C=O group (XXIV). The prefix "dehydro-" may mean also that a hydrogen atom has been removed from each of two adjacent carbon atoms with establishment of a double bond. This use was illustrated in the name "dehydroisoandrosterone" (Fig. 7, XVII). By reference to Table 2 and formulas XXII to XXIV (Fig. 9) the structure of the other hormones can be constructed readily.

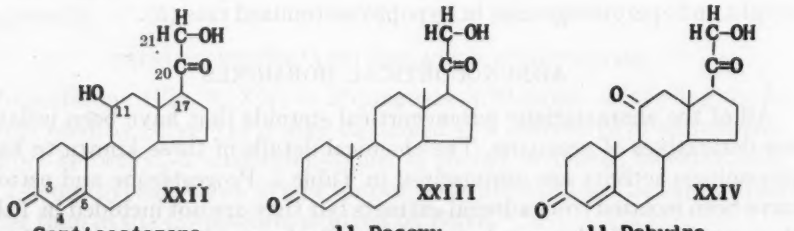
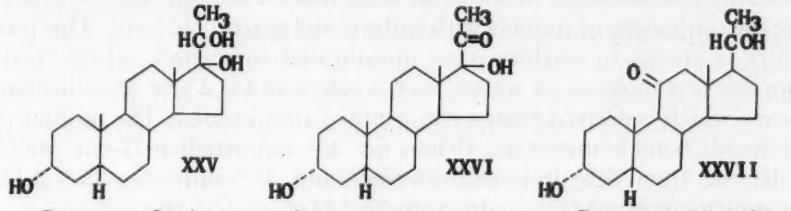


FIG. 9

11 Desoxycorticosterone (usually named simply "desoxycorticosterone") acetate is used widely for therapy in Addison's disease. In this compound a molecule of acetic acid has been combined with the OH group at C-21 to give an ester. This compound could properly be named "11-desoxycorticosterone 21-acetate."

Many other compounds have been isolated from adrenal extracts but only those that are known to have physiologic activity are considered here.

In all of the adrenal steroids, including those that are not active hormones, if a hydroxyl group is present at C-11 it has the (β) configuration. Also, the side chain at C-17 has the (β) configuration. Therefore the 17-hydroxyl group in 17-hydroxycorticosterone, 11-dehydro-17-hydroxycorti-



Pregnane-3(α),17,20-triol | Pregnane-3(α),17-diol-20-one | Pregnane-3(α),20-diol-11-one

FIG. 10

costerone and 17-hydroxyprogesterone must have the (α) configuration. A more systematic name for 17-hydroxycorticosterone would be "Δ^4-pregnene-11(β),17(α),21-triol-3,20-dione," or, according to the system used by Selye, "17(β)-(1-keto-2-hydroxyethyl)-Δ^4-androsten-3-one-11(β),17(α)-diol." The latter name differs from that given by Selye in that the configurations assigned to the side chain and the hydroxyl group at C-17 are reversed in accordance with more recent evidence on the configurations at C-17. Note the order of "-one" and "-diol."

The term "11-oxysteroids" has been applied recently to urinary material which has the ability to stimulate deposition of glycogen in fasting adrenalectomized rats or mice. This property is characteristic of the adrenal hormones which have an oxygen atom at C-11. Although it may be logical to assume that the urinary material which has this type of physiologic activity has an oxygen atom at C-11 it is undesirable to assign to it a name which includes a specific structural feature when the exact nature of the material is still unknown. The terms "corticoid," "corticosterone-like" and "11-oxycorticosteroid-like" have also been applied to the urinary material. These terms imply only that the urinary material has properties like those of some of the adrenocortical hormones.

Probable metabolites of adrenal steroids are pregnane-3(α),17,20-triol (Fig. 10, XXV) and pregnane-3(α),17-diol-20-one (XXVI). Pregnane-3(α),20-diol-11-one (XXVII) (11-ketopregnanediol) is a metabolite of 11-dehydrocorticosterone and pregnane-3(α),20(α)-diol (Fig. 8, XXI) is a metabolite of 11-desoxycorticosterone as well as of progesterone. In XXV and XXVII (Fig. 10) the 20-hydroxyl group probably has the same configuration as in the ordinary pregnane-3(α),20(α)-diol but this has not been established definitely. Also, the OH at C-17 most probably has the (α) configuration but the configuration has not been proved definitely.

ARTEFACTS

Usually it is necessary to boil urine with mineral acids in order to hydrolyze the conjugates of steroids with sulfuric and glucuronic acids. This procedure is known to produce some unsaturated compounds which result from loss of a molecule of water. Such a compound is Δ^2- or Δ^3-androsten-17-one, which is derived from a conjugate of androsterone. The position of the double bond is uncertain. Others are $\Delta^{3,5}$-androstadien-17-one which is derived from dehydroisoandrosterone, and $\Delta^{9,11}$-androsten-3(α)-ol-17-one, which is derived from androstane-3(α),11(β)-diol-17-one.

ESTROGENS

In the estrogens, ring A of the steroid nucleus has become an aromatic ring; that is, essentially a benzene ring with three double bonds. In order

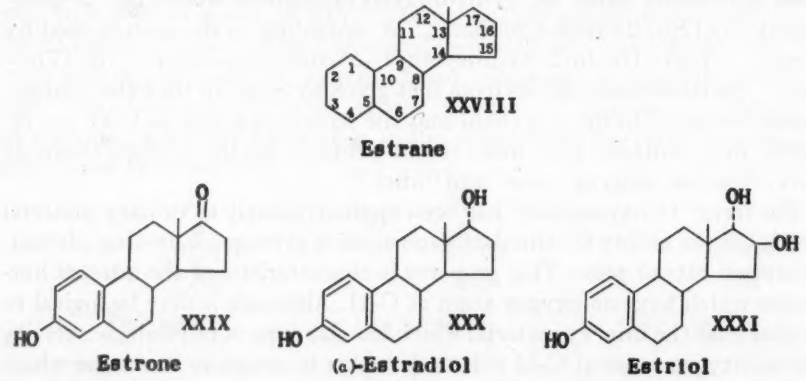


FIG. 11

for this ring to accommodate three double bonds the methyl group at C-10 has been lost. The aromatic character of this ring gives the hydroxyl group at C-3 the properties of a phenolic hydroxyl group. It has weakly acidic properties.

The saturated hydrocarbon which may be regarded as the parent substance of the estrogens is named "estrane" (Fig. 11, XXVIII). It is almost identical with etiocholane but lacks the methyl group at C-10. The numbering system is the same as for etiocholane. Inspection of the formula of estrone (XXIX) shows that it has three double bonds, a hydroxyl group and a ketone group. Its systematic name, therefore, is "$\Delta^{1,3,5:10}$-estratrien-3-ol-17-one." α-Estradiol (XXX) is $\Delta^{1,3,5:10}$-estratriene-3,17(α)-diol and estriol (XXXI) is $\Delta^{1,3,5:10}$-estratriene-3,16(β),17(α)-triol. Equilin and equilenin, respectively, are $\Delta^{1,3,5:10,7}$-estratetraen-3-ol-17-one and $\Delta^{1,3,5:10,6,3:9}$-estrapentaen-3-ol-17-one. The superscripts 5:10 and 8:9 indicate the ends of the double bonds in these positions, since the use of 5 or of 8 alone would be ambiguous.

A more extensive discussion of steroid nomenclature will be found in the monograph by Fieser (7). This subject is also discussed in the books by Gilman (8) and Sobotka (9). Examples of trivial and systematic names of hormones and related steroids will be found in Selye's Encyclopedia (1).

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* This name would now be "17(α)-Hydroxyprogesterone."

FRED CONRAD KOCH
1876–1948

Obituary

★

FRED CONRAD KOCH

1876–1948

FRED CONRAD KOCH died at his home in Chicago on January 26, 1948. Not long before he had been ill with pneumonia but convalescence seemed to be progressing. Death, presumably from a heart attack, was unexpected. Those who mourn him may take some comfort from the circumstance that he was spared the long months of feebleness, disintegration of abilities, pain and anticipation of death that are so common. The image of full and friendly vigor left at our last meetings remains unimpaired.

Professor Koch's entire career developed within his native state. He was born in 1876 in Chicago. He studied at the University of Illinois, obtaining his bachelor's degree in 1899. After two further years there, teaching and working in organic chemistry, he went to Armour and Company as a research chemist. In 1909 his work took a new turn. The formal study of biochemistry was begun on a fellowship at the University of Chicago under A. P. Mathews. He received the Ph.D. degree in 1912. From then on until his retirement in 1941, he was affiliated with this department of biochemistry, serving as acting chairman from 1919 to 1926 and as chairman thereafter. His academic life culminated in 1941 with his appointment to the Frank P. Hixon Distinguished Service Professorship. When his university duties were over he resumed his old association with Armour and Company, serving as director of biochemical research. His career matured slowly but suffered no decline with advancing years. Well merited recognition came late after long arduous effort. He knew no true retirement.

His diversified contributions to endocrine research are well known to all. He will be best remembered, however, by his influence on the development of our understanding of testicular function. In 1926 he guided the work of a graduate student, L. C. McGee, in demonstrating the power of bull-testis extracts to induce growth of the capon's comb. With T. F. Gallagher and others this response was adapted to the quantitative measurement of the androgenic potency of tissue extracts, body fluids and pure steroids. With several others, the details of biological reactions to male hormone and hormone-like substances were described. Methods of extraction of active materials from urine were elaborated and an initial account given of the occurrence of androgens and estrogens in the urine of man. The discovery of androgenic activity in the urine of women was startling in its day and has set a perennial physiological problem. In his later years he was much concerned with pituitary functions and at Armour and Company accomplished the production of adrenotropins of high quality and in sufficient amounts for biological and clinical experiment. He gave attention to many other biochemical matters. With his wife and constant collaborator, Elizabeth Miller Koch, continuing studies on vitamin D were pursued.

Professor Koch was a devoted member of the Association for the Study of Internal Secretions, serving as its president in 1937. He received the coveted Squibb Award in 1942. Many will gratefully recall his tireless attention to the multitude of papers at our sessions, without regard to the greater or lesser fame of the speakers, and his unending courtesy to all in discussions after hours.

Claude Bernard once said, "Art is myself, science ourselves." This Professor Koch understood by temperament and instinct rather than from bitter lessons. As a great teacher he felt his highest honors in the success of his students. Their work now ramifies through many subjects, and much of it deals with endocrinology. From such seeds the harvest is incalculable. Here lies abundant fatherhood. The general sense of loss is tempered now by gratitude for what has been done.

ANNOUNCEMENT OF THE 1948 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting of the Association for the Study of Internal Secretions will be held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

The scientific sessions will be held in the Red Lacquer Room and registration will be on the fourth floor just outside the Red Lacquer Room. The Annual Dinner will be held in the same room on Friday, June 18th at 7 P. M. and will be preceded by a cocktail party, the location of which will be announced later.

All members of the Association who plan to attend the Thirtieth Meeting are urged to make their reservations at once with the Palmer House, stating the time of arrival and how long they plan to remain in Chicago.

The program of the meeting will appear in the April or May issue of the Journal, and the abstracts of papers presented at the annual meeting will be published in the July issue.